HORMONE ASSAY

Hormone Assay

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PREFACE

The speed with which our knowledge of hormones has increased during the past few decades is illustrated in the recent two-volume survey edited by Pincus and Thimann[1] and by the fact that in that treatise it was possible to give only brief mention of methods of assay. In the present volume, assay methods are considered in detail, yet it has still been necessary to confine them to vertebrate hormones and to condense accounts of statistical methods to a bare minimum. Statistics as applied to biological assay is another field in which recent advances have been phenomenal, although the application of modern methods to the design and analysis of many assays awaits full development. This simultaneous progress in the two fields of work has nevertheless provided a fruitful series of examples of how assays can be conducted in order to gain the greatest amount of information from the material available, and to express results in precise form.

Chapter I gives a brief account of the use of and need for statistics in assays, and is followed by a chapter on insulin, which exemplifies some of the methods described. It is not possible to maintain a high level of statistical treatment throughout the greater part of the work, because the majority of hormone assays have not been examined in as detailed and often not in as precise a manner as has been possible in the case of insulin. The various contributors have therefore been forced, by the nature of their material, to present many methods of less certain accuracy, particularly since they were asked to give up-to-date accounts of any ways of assaying hormones that seem promising, even though sometimes of uncertain eventual value. The scope of the work has, however, been limited to procedures which in some measure merit the word "assay," so that purely qualitative tests aimed at detecting the presence or absence of a hormone and not giving an estimate of the amount present are omitted, unless there is good reason to believe that they can be developed into an assay technic.

I should like to take this opportunity of publicly thanking the contributors to this volume for their ready and prompt co-operation at all stages of production. I moved to Australia after having discussed the projected book with a number of them, and although I was by odd chance accompanied by two other contributors, matters were not made easier for the remainder or for the Academic Press. The understanding and helpful attitude of the latter has made it a pleasure to produce this volume.

C. W. EMMENS

Sydney, Australia
  September 1950

[1] The Hormones, Physiology, Chemistry and Applications. Edited by G. Pincus and K. V. Thimann, Academic Press Inc., New York, Vol. I, 1948; Vol. II, 1950.

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Statistical Methods

BY C. W. EMMENS

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I. INTRODUCTION

1. The Validity of Assays

It is now widely recognized that a satisfactory assay, whether conducted by biological or chemical methods, gives an unbiased estimate of potency and of the limits of error within which the estimate falls. These limits will be assigned at a stated level of probability; such that in effect

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there is only one chance in 20, or in 100, that they do not cover a range within which the true value lies.   Such an assay is realized only if the design fulfils certain minimal requirements, which help to guarantee the statistical validity of the complete test.   The present chapter is concerned mainly with biological assays, in which the need for statistical control is imperative; but many of the arguments apply with equal force to chemical or physical methods, particularly when they deal with material of living origin.

When the relative potency of two preparations is determined by biological assay, this determination is valid only if:

1. The substances are shown to have similar actions on the test material.   In an assay involving the plotting of log dose against response this is checked by determining the parallel nature of the individual dose-response lines.

2. All responses are obtained simultaneously or are so spaced in time that any secular variation in response can be eliminated in the analysis.

3. The living material is allotted to dosage groups in such a way that the inequalities it may exhibit will not bias the result or the estimation of error.

4. An estimate of error is available from the internal evidence provided by the assay.

The commonest source of uncertainty in assays is lack of attention to (2) above.   Dose-response lines for preparations under test are frequently constructed from data collected at different times and compared with one another as if this condition were unimportant; or a standard curve is made and then subsequent single dosage groups are used to read off the potencies of materials under test.   All such procedures give estimates of potency which are probably biased and are of indeterminate error.

In assays of the type discussed in this volume, it is most frequently found that the line relating log dose to response (or to a suitable transformation of the response) is straight.   This is so common a finding that only this case will be discussed in detail, as it covers practically all of the assays described by the various contributors.   When this is so, conditions (1) to (4) are satisfied by the use of the so-called 4-point assay, in which two doses of each preparation under test are given, each to a group of test objects.   There is usually no point, in such an assay, in failing to keep the number of test objects per group constant, and in failing to keep the ratio of the high to low dose of each preparation the same.   This leads to a very simple form of analysis, particularly when graded responses are available; in which the maximum of information is given by a minimum amount of computation.

2. Standard Preparations

Secular variation in sensitivity, and sometimes in the slope of dose-response lines, has been touched upon in the preceding paragraphs. It is the cause of the failure of "animal units" as a basis for the comparison of activities of drugs and hormones. It causes some surprise to the uninitiated that a stock of animals kept under apparently constant conditions can vary so widely in its response to a standard drug as is commonly found; thus Burn (1937) shows how the frog unit for digitalis varied in the course of a year from 1310 units/g. to 2940 units/g., and Emmens (1939a) how the mouse unit for estrone varied in a similar period of time between 0.064 μg. and 0.150 μg. Recognition of this source of error has led to the adoption of standard preparations, kept under conditions designed to preserve them unchanged, so that they can be used as stable reference materials with which to compare the potency of other substances. International standards for many hormones are available and are distributed to various laboratories in which local substandards should be carefully calibrated in terms of the master standards.

In performing hormone assays, any substance under test should always be compared with a standard. Theoretically, if the test and standard preparations act in a similar manner, an estimate of relative potency derived from their simultaneous comparison should be independent of the test object and of the particular response utilized for the test. In practice, differences sometimes occur when the same two substances are compared by using different animals or criteria of response, even when statistically valid assays are performed. These discrepancies may be caused by impurity or inhomogeneity of one or both preparations, or by such factors as different rates of absorption or utilization under different test conditions. Striking examples are given in Chapter XVI, which deals with estrogen assays.

3. Fitting Dose-Response Lines

Although a linear relationship between log dose and response is frequent in hormone assays, it never holds over the whole range of doses that can be given. Emmens (1940) has shown that for many pituitary and pregnancy gonadotropin preparations a logistic curve is a better description of the known range of possible responses, although a log dose-response line fits very well over a wide, useful range. This, and similar findings should not, however, lead us to abandon the latter as an approximation to fact; because it is mathematically very much more convenient and in most cases a perfectly adequate method of graduation. Any collection of

results can be fitted by an infinity of functions; we merely choose a function which, while fitting the facts as well as can be expected, also leads to suitable and simple formulae from which to estimate relative potency and the errors of the test.   Almost the same estimates of potency and of error would be obtained by other, reasonable methods of analysis.   In quantal analysis (see below) Finney (1947) has shown how the same data analyzed by four different methods gave practically identical estimates of potency and error.   These are listed in Table I, which gives the relative potency of digitalis injected to frogs by two different routes, its potency when given into the lymph sac being less than when given intramuscularly.   The data are those of Miller *et al.* (1939).

TABLE I

The Relative Potency of Digitalis in Frogs When Injected by Two Different Routes
(Intramuscularly and into the Lymph Sac) as Calculated on Different Assumptions.
Adapted from Finney (1947)

Transformation of response	Relative potency	Limits of error
Probit	2.09	1.70–2.65
Logit	2.09	1.69–2.67
$P = \sin^2 Y$	2.08	1.70–2.62
$P = Y$	2.06	1.72–2.53

We see, therefore, that a certain degree of mathematical fiction need not disturb us unduly in the interpretation of biological assays; the logical arrangement of tests and the use of efficient statistical methods are far more important than the precise validity of any particular assumption about the nature of the dose-response line.   Any significant departure from the assumption made will, of course, be revealed in the analysis and lead to a recalculation along different lines.

4. Approximate and Graphical Methods

The present writer must confess to a prejudice against short-cut approximations.   They abound in the literature, particularly that dealing with quantal responses.   They have a place in routine work where it is sometimes a burden to make frequent calculations, and where precise estimates may be unimportant.   In research, they have little to recommend them.   Approximate methods give no better estimate of relative potency than a simple graphical fitting in the hands of a careful worker, many of them give a worse estimate, and some actually take longer to perform than a full analysis.   The most serious drawback is, however, that such methods give false limits of error (or none at all), which may be so misleading as to invalidate the conclusions drawn with their aid. Fiducial limits of error should be calculated as a research routine in all but

trivial instances, and there is no short cut available for determining them. As this determination necessarily involves the estimation of relative potency itself, the use of more approximate methods is superfluous.

Fiducial limits of error for most types of test were not calculated prior to an investigation by Irwin (1943), and thus some publications before that date give apparently exact formulae which are only correct if the slope of the dose-response line is known with relatively little error. The estimate of relative potency is not affected by Irwin's work when made by orthodox methods.

II. Statistical Terminology and Procedure

1. Sampling and Randomizing

The statistical methods which are outlined below rest on the assumption that unbiased samples of test material are used in assays. This means that of all possible test objects that could have been chosen as fulfilling the requirements of the assay, the selection used was taken by a process which guaranteed that any one of those available was as likely to be chosen as was any other. It is further assumed that, in allotting test objects to dosage groups, a similar process has made it as likely that a particular test object shall have fallen into any one group as into any other. This process is called randomization.

The population from which test material may be drawn is often very restricted, and it may be necessary to use all that is available, as when all the 21-day-old rats in a colony are required for an assay. If this is not the case, however, reasonable care should always be exercised to ensure that those excluded are not peculiar in some way which would not otherwise render them inacceptable for the test. On another occasion, these or similar animals might have to be included, and the least of the consequent troubles may then be that the slope or linearity of the dose-response line is modified, or the variability alters. Completely unbiased sampling is often very difficult or impossible, but that should not prevent an attempt to reduce the bias to a minimum.

Once the objects to be used in a test have been selected, their unbiased allocation to groups is even more important. As long as certain criteria such as approximate normality of distribution are satisfied, bias in the selection of the total number of test objects from the parent population may not invalidate an individual assay, but any bias in distribution between dosage groups (or any other integral subdivision of the experiment) will do so. This partitioning must therefore be strictly at random, by a process analogous to writing the number of each object on a card, shuffling thoroughly, and then dealing out into the various groups. In

practice it is usually easier to use tables of random numbers such as those prepared by Fisher and Yates (1947) or Snedecor (1946). Common errors in randomization are discussed by Emmens (1948).

This process of random allocation to groups is frequently practiced within various restrictions in the design of an assay, examples of which are discussed below. In a simple example, there may be a number of litters of animals available, and we may believe that the variation in response within litters is less than that between them, and wish to take advantage of that fact. If one member of each litter is placed at random in each dosage group and if in the subsequent analysis the appropriate steps are taken to segregate the variation attributable to differences between the mean reactions of litters, the estimate of error will be reduced in so far as littermates in fact respond more alike than non-littermates. Such procedures are part of the basic technic designed to reduce experimental error and their exploitation has resulted in very considerable reduction in the limits of error of various tests.

2. Variates, Means, Variances, and Standard Errors

There is no space in the present volume for a detailed account of the statistics of biological assay. It is felt, however, that a useful purpose can be served by a more descriptive treatment in which the advantages of the proper application of statistics are illustrated and in which only the main important concepts are defined. As the biologist's reading of statistical papers is often spoiled by the fact that he is plunged into unfamiliar concepts with little in the way of explanation, it is felt that much is to be gained by including some elementary definitions. The rest is easier than is often supposed.

A *variate* is something that varies. In the case of two variates like the dose and the response in an assay, the dose, which causes the response, is the *independent variate;* the response is the *dependent variate.* The dose is usually denoted by X and the response by Y. The matter is discussed more fully in Section III of this Chapter.

The *arithmetic mean* of a group of observations of a particular variate is indicated by placing a bar over the appropriate symbol; the mean of several responses is $\bar{Y}$. Mathematically,

$$\bar{Y} = SY/n$$

where S is an operative symbol implying the sum of all values of Y, and n is the number of values summed.

There are other than arithmetic means; these do not concern us here, except that it may be noted that the arithmetic mean of the logs of a

series of doses is the log of the *geometric mean* of these doses. (The geometric mean is the nth root of the product of n values of a variate.)

The sum of the deviations of all values of Y from $\bar{Y}$ is zero. These deviations are frequently denoted by small italics, y; hence $Sy = 0$. The sum of the squares of these deviations is minimal; if they are taken from any other point this sum is greater than that which results when the mean is used.

The *variance* is the sum of squares of y divided by one less than the number of values summed:

$$V = s^2 = Sy^2/(n - 1)$$

The symbol V may be replaced by s^2; s is the estimate of the *standard deviation*, σ, the square root of the variance. The divisor, $n - 1$, used in computing the variance is the number of *degrees of freedom* or independent comparisons on which the estimate is based. The mean is based on n values, the variance is based on $n - 1$ values since only $n - 1$ separate and independent differences between n observations are available. If there is only one observation, Y, the mean is Y and the variance is indeterminate. With two observations, Y_1 and Y_2, the mean is $\frac{1}{2}(Y_1 + Y_2)$ and the variance is $\frac{1}{2}(Y_1 - Y_2)^2$, and so on. Clearly, the variance is a measure of the scatter of observations about the mean, independent of the sign of any particular deviation. It is more informative to think, if possible, in terms of the variance rather than the standard deviation when considering sources of variation and their analysis. The inverse of the variance, or *invariance*, is a measure, by definition, of the amount of information supplied by an observation in the group being considered. A member of a group with unit variance supplies one unit of information; a member of a group with a variance of 4 supplies $\frac{1}{4}$ of a unit.

The *variance of a mean* is $1/n$th of the variance of the group to which the mean belongs. This is another way of saying that the mean of a group of n observations tells us n times as much about the population mean as does any single observation:

$$V\bar{Y} = Sy^2/n(n - 1)$$

where $V\bar{Y}$ is the variance of $\bar{Y}$.

The *standard error* of a mean, $s_{\bar{y}}$, is the square root of the variance; thus $V\bar{Y} = s_{\bar{y}}^2$. The term standard error is used to distinguish the quantity from the standard deviation of individual observations.

3. *The Normal Distribution*

It may be shown mathematically that the expected distribution of a series of estimates of such a quantity as the height or weight of the same

object is a bell-shaped curve, shown in Fig. 1.   This was called the *curve of error* or *normal distribution*, and has the equation

$$y = \frac{n}{\sigma \sqrt{2\pi}} \, e^{-x^2/\sigma^2}$$

with points of inflexion at $x = \pm\sigma$.   This distribution has also been encountered frequently in biometry when measuring the heights or weights, for instance, of a population.   Note that the repeated measurement of one individual is to be distinguished from the measurement of many individuals—both may give a normal distribution, but one

←— 68.2 % —→

←——— 95.5 % ———→

←——— 99.7 % ———→
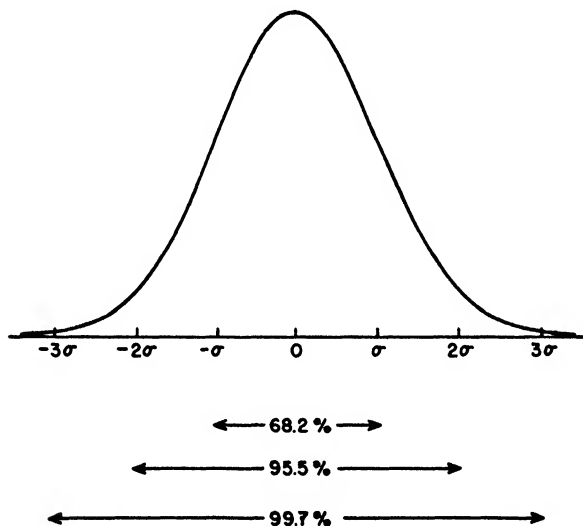
FIG. 1.   The normal distribution.   (From Emmens, 1948.)

arises only from errors of measurement, while the other arises from both errors of measurement and differences between individuals.   When a range of heights in man of from perhaps 4 ft. 6 in. to 6 ft. 6 in. is involved, errors of measurement are unimportant.

The same normal distribution is found when dealing with the responses of living matter to drugs or hormones.   The members of a group on any one dose of a preparation give responses, such as body weight increases or ovary weights, which tend to be normally distributed about their mean. Even when they are not, it is usual to find that a normal distribution sufficiently describes the type of variation encountered to be used in calculations, particularly since the distribution of *means* of samples tends rapidly to normality whatever the nature of the individual distribution.   This is a fortunate occurrence, as it is with means that the statistician is most

concerned in biological assay, and the statistical methods based on the normal distribution can therefore be used with confidence.

There is a second way in which the normal distribution enters into biological assay. Suppose that a group of rabbits is taken and each is injected slowly with curare intravenously until its neck muscles fail and its head drops. By such a method, the individual effective dose can be determined for each animal. When a large number of observations is made in this type of test, it is usual to find that the logarithms of the
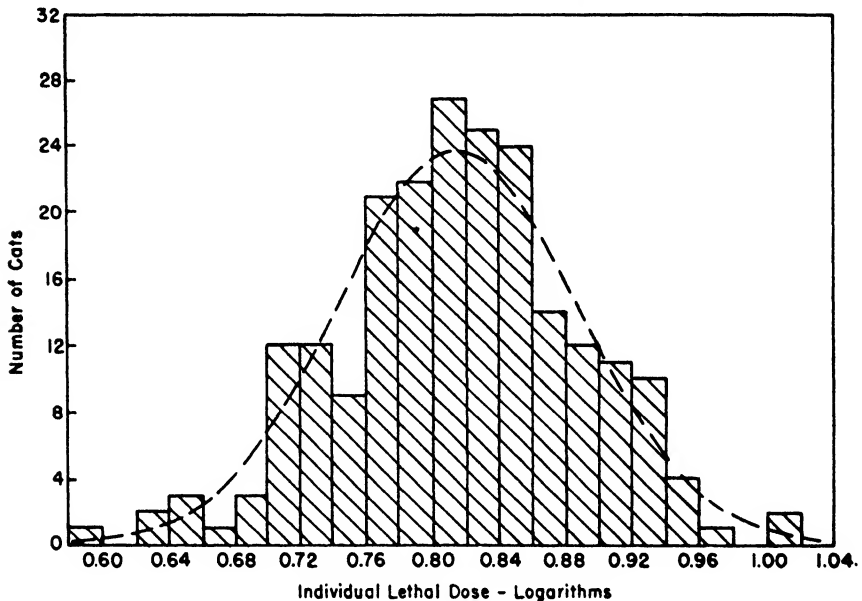
FIG. 2. A normal distribution of individual effective log lethal doses of digitalis in cats. (From Bliss, 1944.)

individual effective doses are normally distributed, not the actual doses themselves. This observation is the basis of methods for dealing with "all or none" (quantal) responses, in which a definite end point is noted as occurring or not occurring; in tests in which it is impossible to give a slow injection and note the individual effective doses as with curare in the rabbit. An example of a normal distribution of individual effective log doses is shown in Fig. 2.

4. Small Samples, t, F, and χ^2

When dealing with large samples from a normally distributed population—samples of several hundreds—the standard deviation may be used with considerable exactness as a means of predicting the proportion of a

sample which will fall inside any given limits with the mean as the central value. Thus, about 68% of all observations will fall within the limits $\bar{Y} \pm \sigma$, where σ is the known standard deviation of which s is an estimate. If s is derived from many observations, it will be a very good estimate of σ, and we could write $\bar{Y} \pm s$. About 95% of all observations will fall between the limits $Y \pm 2\sigma$; and about 99.7% within the limits $\bar{Y} \pm 3\sigma$.

When small samples are used, these relationships vary with the number in the sample, so that tables of a statistic, t, have been prepared in such a form that the proportion of observations falling within the range $\bar{Y} \pm ts$ is easily calculated. Similar calculations are as easily made about the limits of error of the mean of a small unique sample, which can be assigned a range of $\bar{Y} \pm ts_{\bar{y}}$, the value of t again depending on the size of the sample. Table II gives some values of t for small samples and the

TABLE II

Value of t and Limits of Error of a Mean ($\bar{Y} = 100$) with a Population Standard Deviation of 10 When Derived from Small Samples

Sample size	Value of $s_{\bar{y}}$	t	$P = 0.95$ Limits of error	t	$P = 0.99$ Limits of error
2	7.06	12.71	10.3–189.7	63.66	−349.4–549.4
4	5.00	3.18	84.1–115.9	5.84	70.8–129.2
8	3.54	2.57	90.9–109.1	4.03	85.7–114.3
16	2.50	2.37	94.1–105.9	3.50	91.2–108.8
32	1.77	2.04	96.4–103.6	2.75	95.1–104.9
64	1.25	2.00	97.5–102.5	2.66	96.7–103.3
∞	0.00	1.96	. . . .	2.58	. . . .

corresponding limits of error of $\bar{Y}$, supposed to be 100, with an s of 10, for the levels within which 95% and 99% of such determinations will fall. These levels are denoted by $P = 0.95$ and $P = 0.99$ respectively, where $(1 - P)$ is the probability of equalling or exceeding the deviations shown by chance. It will be seen how rapidly the value of t increases as very small samples are taken, so that the limits of error of a mean of two observations are much greater than those of a mean of four observations; if the same population is sampled and no information is available about the variance other than that provided by the sample itself.

The statistic t is applicable to the comparison of two groups, when the *null hypothesis* may be tested by assuming that the two groups of observations have been drawn from the same population. Then the difference between the two means is tested by determining how likely it is that such a difference has arisen by chance, and if the probability is sufficiently low, the hypothesis is rejected. If it is desired to test several groups at once, t cannot be used, and a more general function, F, the *variance ratio* is

employed. If there is a number of values of $\bar{Y}$, each derived from a separate group of observations, the variance exhibited by these means is compared, in effect, with the variance expected from the distribution of the individual observations about each mean. Again, a null hypothesis is tested, and if the variation between the means of groups is greater than can reasonably be accepted as due to chance, it is concluded that the observations do not form part of a single, homogeneous population. The level of probability at which the null hypothesis is usually rejected is at $P = 0.95$ or 0.99. As before, $(1 - P)$ is the chance that the value of F observed is fortuitous; sometimes the complementary value, $P = 0.05$ or 0.01, is used to denote the same probability, but there is never any ambiguity in meaning as the unlikely event is always being considered. Tables of t and F are given in almost any modern textbook of statistics, with examples of their use.

Another statistic in common use is *Chi-squared* (χ^2). It is used when the measurement of Y is discontinuous, notably when it is of the all-or-none type. If a number of samples is available from which estimates of a percentage of reactors can be made, the homogeneity of these samples is tested by χ^2, by a method analogous to the F test. χ^2 is related to the value of F when an infinite number of degrees of freedom is available for the estimation of one of the variances compared; in effect, the calculation of χ^2 assumes that the value of the variance associated with random sampling is known, which in theory is correct.

Thus t, F, and χ^2 are all connected, F being the basic statistic of which t and χ^2 are particular cases. If n_1 is the number of degrees of freedom associated with the greater mean square in an F test, and n_2 is that associated with the lesser mean square; then t^2 is F for $n_1 = 1$; χ^2 is $n_1 F$ for $n_2 = \infty$.

5. The Analysis of Variance

The arithmetic involved in comparing the two variances in an F test usually amounts to a simple analysis of variance. This technic, introduced by R. A. Fisher, is a powerful statistical method of wide application. It consists in splitting the total variation between all observations in a test into two or more separate parts, each attributable to a defined source of variation. The way in which the analysis can be made depends on the structure of the experiment; therefore tests must be suitably designed if full advantage is to be taken of the method.

Table III gives the blood sugar levels in mg./100 ml. of four different groups of rabbits, 7 per group (Emmens, 1948). The *total* sum of squares of all deviations from the general mean of the 28 observations is $S(Y - \bar{Y})^2$ or Sy^2. This can be divided into two sums of squares:

1. That *between groups*, derived from the deviations of group means from the general mean, is $Sn_p(\bar{Y}_p - \bar{Y})^2$, or $Sn_p\bar{y}_p^2$

2. That *within groups*, derived from the deviations of individual values of Y from their own group means, is $SS(Y_p - \bar{Y}_p)^2$, or SSy_p^2

where n_p is the number in group p; $\bar{y}_p$ the deviation of the mean of group p, $\bar{Y}_p$, from $\bar{Y}$, the general mean; y_p the deviation of an observation Y_p in group p from $\bar{Y}_p$; and SS means "the sum of the sums of."

The analysis is kept on a per item basis, according to the identity:

$$Sy^2 = Sn_p\bar{y}_p^2 + SSy_p^2$$

which illustrates the additive property of the two sums of squares isolated in the analysis.

The analysis for the data in Table III is given in Table IV. In this table, the *within groups* sum of squares gives an estimate of the variance to be expected *between groups* if there is no additional source of variation but random differences between rabbits. If breeds of rabbit differ in mean blood sugar level, the variance observed between groups will exceed that found within groups, as in fact it does. From the relevant tables, the value of $F = 599.7/101.2 = 5.93$ is to be expected less than one in 100 times if there is no real difference between breeds, i.e., $P < 0.01$, and it is concluded that the breeds differ in mean blood sugar.

TABLE III

Blood Sugars in mg./100 ml. of Four Breeds of Rabbits, with Seven Individuals per Group (Adapted from Emmens, 1948)

		BREED		
	1	*2*	*3*	*4*
	117	137	135	109
	116	136	122	108
	128	121	135	117
	104	113	138	118
	121	145	131	101
	100	123	134	134
	123	113	140	113

TABLE IV

The Analysis of Variance for the Data of Table III

Source of variation	Formula	Degrees of freedom	Sum of squares	Mean square
Between groups	$n_pS\bar{y}_p2$	3	1,799.14	599.7
Within groups (error)	SSy_p2	24	2,427.71	101.2
	Sy^2	27	4,226.85	

$F = 5.93$; $P < 0.01$ (1 % point is 4.72)

In graded assays, the analysis of variance is frequently used, with the test so arranged that such a factor as differences between breeds of rabbit can be eliminated in comparing the potencies of two preparations under test, and that other sources of variation described below are also isolated and examined.   There are safeguards which must be observed in applying variance analysis, which may be inapplicable if, for instance, the variance is correlated with the level of response to any high degree.   However, it has found such wide application in biological assay that examination of the majority of adequately designed tests is now based upon it. For some of the pioneer work in this field, the various papers of Bliss and his collaborators may be consulted, notably Bliss and Marks (1939a, b), Bliss and Rose (1940), and Bliss (1940).

6. Probits

When quantal data are provided by an assay, such as the percentage of survivors in groups of injected animals, a different approach is needed. Such data must be thrown into a form which gives a straight dose-response line, convenient for statistical treatment.   It is noted above that the logarithms of the individual effective doses are often found to be normally distributed, and when this is so, the appropriate transformation is the probit.   The probit which corresponds to a given percentage of reactors is

$$5 + Y = 5 + (X - X_0)/\sigma_X$$

where X is the log of the dose causing the percentage of reactors in question and X_0 the log dose causing 50% of reactions.   The quantity 5 is added to keep the probit positive under all practical circumstances.

This way of writing the probit is not useful in practice, because X_0 has yet to be estimated.   Instead, the formula:

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{Y} e^{-\frac{1}{2}Y^2} dY$$

is employed, which derives Y, the normal equivalent deviation (Gaddum, 1933) or the probit minus 5, from P, the % reactors.   Then, after fitting the dose-response line, the adequacy of the transformation is checked by a χ^2 test of the "goodness of fit" of the points to the calculated line.

The variance of a probit is unfortunately not constant, being least for a probit of 5 and increasing above and below that value.   This is the reverse of the variance of a percentage, which is maximal at 50% ($\equiv$ probit 5). This inequality of variance makes the analysis of variance inapplicable, and the probit method is rather tedious to use, as it involves successive approximations to the dose-response line and severely limits the flexibility of tests.   Thus, no method is available for eliminating differences between

breeds or litters of animals if they are used together in quantal assays, and no method has been published for reducing the errors of tests by taking advantage of successive responses from the same animal or of simultaneous responses of littermates.

For the earlier work on this transformation, Gaddum (1933) and Bliss (1935, 1938) may be consulted. It may be noted that the probit transformation might well be replaced by a simpler procedure, as the results in Table I serve to indicate, particularly when, as is sometimes the case, it is not found to give a very good fit. However, no extensive investigation of the suitability of other methods has been made, and no results other than those of Finney in Table I seem to be available. The *angular transformation* (cf. Fisher and Yates, 1947) would seem to be worth attention, as it has the great advantage of a constant variance at all levels of response. There is of course no reason to believe that the angular transformation would give a linear dose-response relationship over the whole range of responses, but neither does the probit in some cases, nor the log dose-response relationship used in graded response assays.

III. TYPES OF RESPONSE

1. Quantitative Responses

It is usually best to use a graded, quantitative response when possible, which should give more information per observation than a quantal reaction. It is easier to apply modern statistical design and analysis to quantitative assays. When there is a variety of ways in which the response may be measured or expressed, it is sometimes difficult to decide which of these is preferable. Some will be ruled out by the trouble involved in measuring them, or their obvious unsuitability for other reasons. The ideal response is easy to measure, has a small error and changes rapidly with dose; it also gives either a linear log dose-response relationship or a linear dose-response relationship; or is easily transformed to a function which fulfils this requirement. Thus, a steep, straight line and relatively low variability are desirable, but are rarely found together.

It has been customary to adjust responses in animal tests to a constant body weight, such as crop-gland weight in g./100 g. body weight in the pigeon assay of prolactin. It is usually best to avoid such empirical adjustments; there is instead a technic, *covariance analysis*, by which the proper adjustments can be determined from the internal evidence of the assay. Giving the dose in proportion to body weight is open to similar objections, and covariance analysis should be employed so as to adjust for the effect of body weight instead. Both the method of dosage and the measurement of effect is best kept as simple as possible until

adequate information is available from which the proper adjustment needed, if any, can be determined.

Covariance analysis employs an extension of the analysis of variance by which other variates which are thought to influence response may be examined. Usually, only a second variate besides the dose is considered, such as body weight, which is then called the *concomitant variable*. When it is difficult or undesirable to select animals of uniform body weight for an assay, any regular influence due to this factor may be corrected in the final analysis by reducing the responses to those of animals of standard weight. The calculations are tedious if several variates are to enter into the computations, or if the effect of one variate is complex, but in the usual test a simple linear correction for a single additional factor is adequate and easy to make. By this method, any assumption about the dependence of response on the concomitant variable is avoided and in future tests it may be possible, for instance, to adjust doses to body weight according to the relationship observed; or it may be preferable to continue correcting by covariance as in the earlier trials.

In some tests, such as the rabbit method for the assay of insulin (Chapter II), it is possible to measure both the initial and final state of the test object, in this case the blood sugar content. It does not automatically follow, however, that it is preferable to use the difference between the two as a criterion of response, even when it is apparent that they are correlated. The point is discussed by Emmens (1948), where it is shown that there must be a sufficiently high correlation between initial and final readings to compensate for the additional variance introduced with the initial reading for the method to be worth while. Thus, as a rather extreme example, in the rabbit method for assaying insulin, it is customary to express the response as the mean percentage fall in blood sugar over several hours following injection, then to correct for initial blood sugar values by covariance analysis. Data of Marks, quoted by Emmens (1948), showed that the use of final blood sugar readings alone, at the second hour after injection, gave as precise a result. It would seem that the introduction of initial blood sugar measurements into the response serves only to increase error; that this increase can be removed by covariance analysis, bringing the error down again to what it would be without this involved procedure. It is possible that covariance analysis to adjust final blood sugars directly for initial values would give a further reduction in error; this has seemingly not been tried.

2. Qualitative Responses

Generally speaking, qualitative responses are a nuisance. When a response can be placed in one of several different grades, such as the

— to + + + + so popular with serologists, it is difficult to handle results statistically. Sometimes, by a lucky fluke, the grades represent nearly equal steps in response and the variance at different levels is the same, or sufficiently homogeneous, and the response can then be treated as though it were a continuous variate—if it gives a large enough linear segment. This happens rarely, and it is not often possible to treat the data in this way.

The commonest qualitative data are quantal, when they can be analyzed as described above, but an alternative and preferable variate is sometimes available, the reaction time. This is relatively seldom used in hormone assays and would repay further work in those cases where individuals receiving doses over the minimal effective dose react with a speed which varies with the dose injected. An example is the survival time of adrenalectomized rats or drakes (Chapter XIV). Another example (Calloway *et al.*, 1942) is the assay of melanophore-expanding hormone based on the time required for the melanophores of hypophysectomized frogs to return to the contracted state. In this assay, time was related linearly to log dose; but in other assays log time has been used in conjunction with log dose to get a straight line. The advantage of using reaction times is that a quantal response has been avoided, and the various designs available for quantitative assays can be used with a variable which gives more information per test object. The disadvantage may be that an awkward range of times, such as 10–30 hr., may be encountered, which entails all-night sessions. It is noteworthy that most assays reported use either a short period in which all observations are made, or a relatively long period of several days, in which errors in observation due to missing the exact time of reaction are not so important.

3. Dose-Response Lines and Transformations

Some mention has already been made of the various types of dose-response line that may be encountered in assays, and of the desirability to reduce those used in computation to a few standard forms. It has also been pointed out that, as long as a line is a reasonable description of the dose-response relationship, the calculated potencies and limits of error will be much the same whatever method is used.

The useful forms for quantitative assay have the following characteristics:

1. *Linearity* of a sufficient segment when the response or a practicable transformation of it is related either to the log dose or the dose itself.

2. *A constant variance* at all levels of response used.

3. *A steep line* in relation to the magnitude of the variance, i.e., the quantity s/b should be minimal.

Transformations of the response are aimed at achieving (1) and (2), most frequently the latter. When the variance is correlated with the response, the standard deviation is often a fairly constant fraction of the response, i.e., the *coefficient of variation* is constant. Then, the use of log response will rectify the variance, but unfortunately it often causes trouble by producing a curved dose-response line. The square root of the response has also been used and might be expected to have a uniform variance when the response is dependent on such factors as cell counts. If no suitable transformation can be found, the fitting of the dose-response line with unequal variances should in theory follow a series of approximations similar to that used in probit assays; but it is fortunate that, in well-balanced assays, when the potency of the unknown has been so well judged beforehand that the responses to it and to the standard are much the same, quite large inequalities in variance may be ignored and there will be little or no bias in the assessment of relative potency. This may not be true with other conditions, particularly if a complex design such as the Latin square has been used.

The value of s/b (sometimes called λ) in various log dose-response assays is a direct guide to their precision; the amount of information per test object is proportional to b^2/s^2. This quantity varies greatly from one method to another and much of the preliminary work in finding a suitable assay method for a given hormone may be concerned with discovering a technic which gives a high-enough value. Thus, in the estimation of adrenal cortical extracts having effects on the metabolism of carbohydrate, Eggleston *et al.* (1946) found that if an increase in liver glycogen was used as the criterion of response the log dose-response line was so much gentler in slope than if the total fermentable sugar was measured that each animal contributed only one-tenth of the information obtained by the latter method. When different methods of measuring or expressing the end point are available, it is always a good plan to explore them as thoroughly as possible, since in biological work such large differences in result can occur as a consequence of small changes in method.

In quantal assays, the same arguments hold except that by the usual probit method equality of variance is not expected and the calculations are more complicated. The most important factor is the steepness of the dose-response line alone, as the variance of satisfactory assays is supplied by theory and checked by χ^2 tests. However, the possibility of converting a quantal assay to a graded assay should always be borne in mind and the change made if possible.

When the response is linearly related to dose (*not* log dose), a situation hardly ever encountered in hormone assays to date, a special technic should be used. This will not be treated here, as it is so rarely needed,

but it is of growing importance in microbiological assays.   Finney (1945), Wood (1946), and Wood and Finney (1946) have described the method in detail.   It is of interest that more accurate determinations of relative potency are possible when the linear dose-response relationship holds down to zero dose than when it does not, as a "common-zero" assay is then possible.

IV. ASSAYS BASED ON QUANTITATIVE RESPONSES

1. Balanced Assays

The simplest type of valid assay is the 4-point design with equal numbers of observations per dosage groups and equal spacing of log doses on the standard and test preparations.   When this is done, the calculations are simple; their basis is discussed by Irwin (1937) and by Bliss and Marks (1939a, b).   If there are n_p test objects in each group, the error of the assay is derived from an estimate of s based on $4(n_p - 1)$ degrees of freedom, namely the *within-group* variance.   If the total of responses in the four groups are S_1, S_2, U_1, and U_2 for the low and high doses of standard and test (unknown) preparation respectively, the remaining three degrees of freedom can be isolated by factorial analysis; these are the components of the *between-group* variance:

1. The difference between substances is represented by the total difference in response to the two doses of each preparation:

$$(U_1 + U_2) - (S_1 + S_2) = 2D \tag{1}$$

2. The slope of the combined dose-reponse line is represented by the total difference in response between high and low doses:

$$(S_2 + U_2) - (S_1 + U_1) = 2B \tag{2}$$

3. The difference between the slopes for the standard and test is represented by the difference between the estimates of the individual slopes:

$$(U_2 - U_1) - (S_2 - S_1) \tag{3}$$

To each of these is attributed a standard error calculated from the value of s, by which their significance can be evaluated.   The assay is invalid if equation 3 is large enough to make it unlikely that the two dose-response lines are the same in slope.

The slope of the combined dose-response line b is such that

$$b = (S_2 + U_2 - S_1 - U_1)/2In_p \tag{4}$$

where I is the log dose ratio, or the difference between log doses, and is the same for each substance.   The precision of the assay depends on

b and s. The log ratio of the potency of the unknown to that of the standard is represented by M, when

$$M = \bar{X}_s - \bar{X}_u + (\bar{Y}_u - \bar{Y}_s)/b, \tag{5}$$

where $\bar{X}_s$ and $\bar{X}_u$ are the mean log doses of the standard and unknown respectively and $\bar{Y}_s$ and $\bar{Y}_u$ are the mean responses to the standard and unknown, all groups combined. This equation is true whatever the number per group or the number of dosage groups. With two dosage groups per substance and the same doses of standard and unknown, the value of M reduces to ID/B. Thus, if it is assumed that one "unit" of the unknown is equal to one unit of the standard, the expression for M is very simple. The antilog of M is then the number of units of the standard required to give the same response as one unit of the unknown, or the relative potency, R.

The standard error of M, s_M, is given approximately by the formula

$$VM = s_M{}^2 = s^2 I^2 (B^2 + D^2)/B^2 \tag{6}$$

This quantity is used in conjunction with t, based on $4(n_p - 1)$ degrees of freedom, so that the limits of error of M are

$$(M + ts_M) \quad \text{and} \quad (M - ts_M)$$

where t is taken at any required level of P, usually $P = 0.95$ or 0.99. The limits of error of the relative potency are then given by the antilogs of the two limits for M; thus the lower limit is always nearer to the estimate of relative potency R than is the upper limit. This method of determining limits of error is not sufficiently accurate unless the value of b is known with little error, so that b/s_b exceeds about 8. The method for examining the ratio of b to s_b will be described later. If it is smaller than 8, *fiducial* limits of error must be separately calculated as indicated below.

When more than 2 doses are used per substance, the arithmetic is still simple, but requires some modification. If 3 dosage levels are employed, there are 6 dosage groups in all and therefore 5 degrees of freedom which can be isolated in factorial analysis. In addition to components (1) to (3) above, components (4) and (5) can be isolated so that:

4. The possible departure of the combined dose-response line from linearity, and

5. The possible opposed curvature of the two separate curves can be examined.

Details of such isolations are given by Bliss and Marks (1939b) and by Emmens (1948), together with full examples of computational methods.

It is not usually necessary to employ more than 2 or 3 dosage groups per substance, but where it is desirable, an extension of the same methods is used. Equation 5 is unaltered, but equations 1, 2, 4, and 6 are modified to take account of the larger numbers of groups. As long as components (3) and above are insignificant, the modified forms of (1) and (2) are always used to form estimates of D and B respectively, when equations 4 and 6 become

$$b = B/I \sqrt{n_p Sk^2} \tag{4a}$$

for an odd number of dosage groups and

$$b = 2B/I \sqrt{n_p Sk^2} \tag{4b}$$

for an even number of dosage groups, where Sk^2 is the sum of the factorial coefficients (Bliss and Marks, 1939b) used in analysis. Then,

$$VM = s_M^2 = s^2 K^2 I^2 (B^2 + D^2)/B^2 \tag{6a}$$

where K is a constant which depends on the number of dosage groups, and the limits of error are calculated exactly as before.

An example, modified from Bliss and Marks (1939b) is given in Table V. There are 8 rabbits per group in the estimation of the potency of a

TABLE V

An Assay of the Potency of Insulin (Adapted from Bliss and Marks, 1939b)

	Standard (units)			Unknown (mg.)		
	· 0.25	0.5	1.0	0.8	1.6	3.2
	11.2	16.5	32.7	19.8	37.7	45.4
	21.2	23.2	14.0	21.7	40.7	28.6
	18.7	25.6	28.9	26.1	29.3	50.4
% fall in	2.8	12.7	40.2	32.2	48.1	47.7
blood sugar	27.2	39.8	35.1	28.5	45.6	50.0
	25.1	28.4	36.2	20.2	35.3	12.4
	25.8	40.0	37.8	35.7	14.2	39.0
	2.2	2.4	39.4	26.1	7.9	38.1
Mean fall	16.8	23.6	33.0	26.3	32.4	39.0

sample of insulin in terms of the standard; 3 dosage levels are used with each substance and each dose is twice that preceding it. The response is the mean % fall in blood sugar over a 5-hr. period following injection, a rather elaborate response, as remarked on p. 15. The analysis of variance is given in Table VI where it may be seen that a highly significant slope has been obtained, i.e., the assay is valid, for if there were no certainty about the slope of the dose-response line the basis of the assay would itself

be doubtful. Departures from linearity and parallelism of the two separate dose-response lines are negligible; in fact they are smaller than could reasonably be expected with an error variance of 125.9. The analysis also shows that there is probably a difference in potency between corresponding doses of standard and unknown, i.e., 0.8 mg. of unknown is probably more potent than 0.25 unit of standard (see Table V).

TABLE VI

Analysis of Variance for the Data in Table V

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Between samples	1	780.9	$780.9 = D^2$	6.2	<0.05
Slope of D/R line	1	1673.3	$1673.3 = B^2$	13.3	<0.001
Departure from parallelism	1	25.9	25.9	....	.....
Curvature (combined)	1	6.8	6.8	....	.....
Curvature (opposed)	1	3.0	3.0	....	.....
Error	42	5286.8	125.9		
Total	47	7776.7			

The modification of (5) to be used in determining M, the log ratio of potencies when 3 dosage groups are used per substance is

$$M = (\sqrt{8/3}ID)/B$$
$$= \sqrt{8/3} \times 0.3010 \times \sqrt{780.9}/\sqrt{1673.3}$$
$$= 0.336$$

Hence the log of the potency of 3.2 mg. of the unknown (assumed equal to 1 unit of standard in the set-up of the test) is 0.336, antilog 2.17. Hence 3.2 mg. is equivalent in action to 2.17 units; 1 mg. = 0.68 unit.

The standard error of M is approximately as in equation 6a, where $K = \sqrt{\frac{2}{3}}$, whence $s_M = 0.167$. The value of t for 42 degrees of freedom at $P = 0.95$ is 2.02; whence the limits of error of the determination of potency are the antilogs of $0.336 \pm 2.02 \times 0.167$, or 1.00 and 4.71 units approximately per 3.2 mg.; 0.31 to 1.47 units per milligram. The *percentage* accuracy at $P = 0.95$ is thus 46–218% approx., and this calculation is only approximate, as is noted below.

With unbalanced assays, calculation is more tedious, and the yield in information is less. They can usually be avoided, and even the loss of one or two observations in an otherwise balanced test does not preclude the application of the methods outlined above. Missing values can be supplied from the internal evidence of the test, allowance being made for the inevitable slight loss in precision in the estimation of error (cf. Snedecor, 1946; Emmens, 1948).

2. Methods of Reducing Error

Beside the obvious ways of reducing the value of s to a minimum by such measures as close approximation to uniformity of animal stocks or other living material, including the use of pure lines, uniformity of test conditions and of any subjective elements that may enter into the test, there are methods of statistical control which have the great value that they can be operated in the presence of considerable heterogeneity in the test objects. Covariance analysis has already been mentioned and is a valuable measure whenever a concomitant variable takes a continuous or even discontinuous but measurable form. When the living material in the test is heterogeneous, yet the heterogeneity which it is desired to examine cannot be assigned to a scale such as is provided by body weight or age, other methods are used. The animals in a test may consist of several identifiable litters or strains, or the same animal may be usable several times. Whenever the test objects can be divided into several groups which are believed or are known to be more homogeneous than the population as a whole, or whenever the same test object can be used more than once, various restrictions in the design of tests may be introduced so that advantage may be taken of the greater similarity in response within subclasses. This, as will be seen, does not necessarily mean that the same dose must be given to members of the same subclass.

In a simple instance, there are several litters available for a test each with sufficient members to cover all dosage groups. Then a member from each litter is placed on every dose level of standard and unknown, so that there are as many test objects per group as there are litters. Since each litter, regarded as a unit, has received all possible doses of each substance, differences between the average response of litters are measured by the corresponding totals for litters; and these overall differences can be eliminated from the estimate of error. If there are 8 litters and 2 doses of each substance are given, there are 32 animals in the test. Of the 31 degrees of freedom involved, 7 are associated with differences between litters, 3 with differences between doses—to be split up as in Section IV, 1—and the remaining 21 are available for the estimation of error.

In the assay of insulin, as described above, the same rabbits may be used repeatedly, and differences between individuals are commonly segregated in the analysis. In a typical example, Bliss and Marks (1939b) found the mean square between rabbits to be 845, while that within rabbits (= error) was only 41.4. Had the between-rabbits sum of squares been mixed in with the true error term as determinable in an adequately designed test, or had a between-rabbits sum of squares only been available, the estimate of error would have been much inflated.

A popular example of the type of restriction under discussion is the Latin square, first used extensively in agricultural research. If a square field of crops is divided, like a chessboard, into a number of smaller squares, the yield per acre in each small square will usually be more uniform than over the field as a whole. There may be a gradient of fertility from one side of the field to another, or strips of more fertile land running across the field. Such irregularities are examined by so arranging experimental plots that each treatment occurs once in each row and column of small squares, as in Fig. 3, which shows a square with 6 rows, 6 columns, and 6 treatments, A–F. The number of treatments must be equal to or a factor of the numbers of rows and columns. Then, since each row or column contains an example of each treatment, the total yield in it will not be biased by treatment effects, and differences between these totals represent differences between rows and columns. If fertility effects are large, these differences will be greater than can be accounted for by errors of sampling over the surface of the field.

C F A B E D
B E C F D A
D B F C A E
E D B A C F
F A E D B C
A C D E F B

Fig. 3. A 6×6 Latin square.

In assays, the physical layout of rows and columns in a field is usually replaced by arrangements involving a balance between litters, times of injection, etc. Sometimes the purely physical Latin square may be used, as in arranging cages in racks in an animal house, but it is more usual for each and every dose level to be administered to, say, one member of a litter and one cage of rats, litters being distributed between cages. Such arrangements must retain randomization, both in the selection of the appropriate Latin square and in the allocation of test objects to treatments. This randomizing is performed, however, within a restricted scheme which, while permitting elimination of unwanted sources of variation, still fulfils the condition that each test object is as likely to receive any dosage level of standard or unknown as any other dosage level. When the same test object can be used repeatedly, a Latin square arrangement may be employed so that each test object is treated at each dose level of both compounds and all dose levels are tested on each occasion, doses being otherwise allotted at random to test objects and times of testing.

Each cell of a Latin square may contain one or more test objects. Usually, several would be used per cell in biological assay, so that a *within-cell* variance could be used in estimating error. If not, the residual variance, which consists of the various interactions between rows, columns, and treatments must be used as error. In a 6x6 square,

there are 35 degrees of freedom in all. Five of these are associated with differences between rows, 5 with differences between columns and 5 with differences between doses (a 6x6 square would have 3 dose levels of each preparation). There remain 20 degrees of freedom for the estimation of error, an ample number in many tests for a sufficient approximation to be made to the error variance. In the general run of biological tests, interaction is negligible, and the estimation of error without the advantage of within-cell replication is adequate. Its adequacy should, however, be checked if possible, as the use of significant interactions as error may bias the estimates because of the possibility of heterogeneity in the sum of squares used as error.

Different Latin squares may be used simultaneously, as in the examples by Bliss and Rose (1940), in the estimation of parathyroid hormone by blood calcium changes in dogs. Bliss and Rose used a series of 4x4 Latin squares in 2x2 assays, so that 12 dogs were used in all, each at every dose level upon different occasions.

3. More Complex Assays

The complexity in design of agricultural field trials is sometimes imposing, and no comparable data seem to have been produced in work with animals or in biological assays. This is partly because the object of the work is often different from the agriculturalist's objects, but partly because the technics already available have not been explored fully in assays. There are as yet only a few scattered examples in the literature of assays planned with any pretense to boldness and vision in this respect, or of animal trials in which anything like full advantage of modern ideas is taken.

Balanced incomplete block designs afford considerable scope, so far only investigated to any extent in the simplest form of *symmetrical pairs*. When the material at hand is segregable into very small groups, such as small litters, or twin pairs, it will perhaps be impossible to give every dose level to every individual. When this is the case, differences between groups (blocks) can be eliminated as long as every possible combination of all doses is given to the same number of groups. Assays using sym-

TABLE VII

An Assay Scheme with Symmetrical Pairs

Pair No.	Doses	Pair No.	Doses
1	U_1 and U_2	7	U_1 and U_2
2	U_1 and S_1	8	U_1 and S_1
3	U_1 and S_2	9	U_1 and S_2
4	U_2 and S_1	10	U_2 and S_1
5	U_2 and S_2	11	U_2 and S_2
6	S_1 and S_2	12	S_1 and S_2

metrical pairs have been described by Bliss and Rose (1940). A 4-point assay requires multiples of 6 pairs of test objects, an arrangement such as that in Table VII would give 6 responses to each dose level. With triplets instead of pairs, multiples of the scheme given in Table VIII could be used, each unit providing 3 responses to each dose level. The analysis of such designs is not simple and professional assistance is advisable.

TABLE VIII

An Assay Scheme with Triplets of Test Objects

Triplet No.	Doses		
1	U_1	U_2	S_1
2	U_1	U_2	S_2
3	U_1	S_1	S_2
4	U_2	S_1	S_2

Cross-over tests were first used in the assay of insulin, which still provides the neatest example in the twin cross-over test of Smith *et al.* (1944). The test is described in more detail in Chapter II. Its neat feature is the confounding of differences between rabbits and differences between dose-response lines (departure from parallelism) in a test in which the slope of the dose-response line is almost certainly known to be the same for the standard and unknown, both being pure or nearly pure insulin. A more accurate estimate of the value for the combined slope and differences in potency is available from the within-rabbits sum of squares, while a difference in combined slope on the 2 days in which the test is completed does not affect the assay.

The 4 groups, each of at least 3 rabbits, receive doses as in Table IX, the highest dose of the standard and the lowest dose of the unknown are "crossed over" in 2 of them and the reverse cross is made in the other

TABLE IX

Arrangement of the Twin Cross-over Test

Group	1st Day	2nd Day
1	S_2	U_1
2	S_1	U_2
3	U_2	S_1
4	U_1	S_2

2. Differences between days are therefore eliminated in the analysis, while a difference in the number of animals per group does not affect the working of the particular test.

The results are arranged as in Table X and computation proceeds in terms of the sums and differences, Y and y, of responses from the same group. Comparisons which can be built up from the y's are *within-group* comparisons and have a variance dependent upon Vy, estimated from the differences for individual animals within groups, with $(Sn_p - 4)$

degrees of freedom:

$$M = ISy/(y_2 + y_3 - y_1 - y_4) \qquad (7)$$

where I is the log dose interval. Departure from parallelism of the two separate dose-response lines for the standard and unknown is measured by a quantity built up from the Y's, viz.,

$$(Y_1 + Y_4) - (Y_2 + Y_3) \qquad (8)$$

This has a variance $(VY)\left(S\dfrac{1}{n_p}\right)$, with VY a *between*-rabbits variance, also based on $(Sn_p - 4)$ degrees of freedom.

TABLE X

Analysis of the Twin Cross-over Test

Group	No. of animals	Mean response to		Sum	Difference
1	N_1	S_2	U_1	Y_1	y_1
2	N_2	S_1	U_2	Y_2	y_2
3	N_3	S_1	U_2	Y_3	y_3
4	N_4	S_2	U_1	Y_4	y_4

Estimation of the limits of error of M is described in the next section, since it is easy to calculate direct fiducial limits of error, and examples of the use of such methods are given in Chapter II.

Groups of tests may combine the estimation of potency of several substances at once, or may be spread out in time so as to estimate accurately the potency of a single substance in several successive assays. In the first instance, the simultaneous assay of several unknowns, advantage may be taken of a single series of doses of the standard, but for maximal efficiency in simple assays not involving the segregation of within-animal or within-litter sums of squares there should be $\sqrt{N}$ times as many test objects on each dose of the standard, where N is the number of unknowns. With more complex designs, it is easier to plan tests with equal numbers in all dosage groups.

4. Fiducial Limits of Error

Although a few authors, in isolated instances, calculated exact limits of error in biological assays prior to Irwin's (1943) paper, it was not commonly realized that the formula utilizing s_M and t as above is misleading when the value of b is not large in comparison with its standard error. The approximate formula gives finite limits of error in cases where, for instance, the slope is not itself significantly different from zero at a given level of probability. Clearly, the true limits when this is so must be zero to infinity. Such accurately calculated limits, which take

fully into account the uncertainty in the value of b are called *fiducial limits of error*, to indicate their greater precision. Bliss (1946) refers to them as *confidence limits*, in an important paper giving methods of calculation when factorially designed assays are used. They must be separately calculated for each P value required.

The calculation of fiducial limits for the assay of insulin on p. 20, for which the approximate limits were 46-218%, gives new limits of 53-374%, which are much wider than the approximate limits and also cover a different range. The estimate of relative potency is not, however, affected; only the confidence we have in its reliability is changed. Here the value of b/s_b is only 3.6; and a value of 8.0 or more is needed to make the exact calculation at the $P = 0.95$ level superfluous. Irwin gives a table in which a number of such comparisons are made; his results are shown in Table XI. They refer to determinations of the dose of vitamin E required for fertility in rats.

TABLE XI

Comparison of Fiducial and Approximate Limits of Error for the Median Fertility Dose of Vitamin E (Modified from Irwin, 1943)

| Slope divided by its S.E | $P = 0.95$ | | $P = 0.99$ | |
	Fiducial %	Approx. %	Fiducial %	Approx. %
2.74	19–143	58–172	0–155	49–204
2.76	72–117	85–117	24–127	81–123
3.64	53–128	72–139	29–136	65–155
3.76	80–128	82–123	71–148	77–131
3.90	76–122	82–123	63–131	77–131
3.98	83–119	85–117	75–129	81–123
4.03	83–123	84–119	77–137	80–125
4.05	83–121	85–118	75–131	80–125
4.74	83–116	86–117	76–122	82–122
5.17	88–116	88–114	83–122	85–118
5.36	85–116	87–116	80–123	83–121

In the twin cross-over test just discussed, fiducial limits of error are calculated as follows:

1. F for 1 and $(Sn_p - 4)$ degrees of freedom is taken from an appropriate table of F at the required level of probability.

2. $U^2 = (y_2 + y_3 - y_1 - y_4)^2 - (FVy)\left(S\frac{1}{n_p}\right)$, a quantity that must be positive if real limits exist at the level of P chosen.

3. $UT = (y_2 + y_3 - y_1 - y_4)Sy - (FVy)\left(\frac{1}{n_2} + \frac{1}{n_3} - \frac{1}{n_1} - \frac{1}{n_4}\right).$

4. The limits are the roots of the equation:

$$U^2m^2 - 2UTIm + T^2I^2 = 0, \text{ solving for } m.$$

Similar calculations for a triplet cross-over test are given in Chapter II.

V. Assays Based on Qualitative Responses

1. Balanced Assays

As with graded response assays, the simplest valid quantal assay is the 4-point design, also with equal numbers per group and equal spacing of log doses. The calculations are not as simple, however, as the variance differs at different levels of response when the almost universal probit analysis is applied. The error variance is supplied by theory, and tested by χ^2. If the χ^2 test indicates that the theoretical variance is exceeded by the data, any single assay is unlikely to give much information, as a heterogeneity factor has to be introduced, with 2 less degrees of freedom than there are groups in the test, leaving only 2 degrees of freedom for the estimation of error in a 4-point assay. As mentioned earlier, the probit transformation does not lend itself to an analysis of variance because of the correlation between variance and response, and no statistical method has yet been described by which the similarity in response of littermates, for instance, can be utilized in reducing error.

Computation when probits are plotted against log dose involves the following steps; irrespective of the number of dosage groups:

1. Two parallel straight lines are drawn by eye, one each to fit the date for the standard and unknown respectively as well as can be estimated. Alternately and probably preferably, simple formula may be used to give good approximations (cf. Emmens, 1948). In fitting these lines, allowance should be made for response of 0 or 100% by altering slightly the slope derived from other points.

2. The provisional lines are then used for reading off the *expected probit* at each dosage level. The observed probits at these levels are called *empirical probits*. From these two sets of probits *corrected probits* are calculated from tables such as Bliss (1938) or Fisher and Yates (1947) supply. These, in conjunction with the corresponding *weighting factors* which allow in the calculations for the variance of the probits concerned, are used to calculate a first approximation to the dose-response lines, which will be parallel in slope but usually not identical in position.

3. If the provisional and first approximation to the slope differ much (by more than a small fraction of the standard error of the slope) a second and perhaps further approximations are calculated by repeating the same cycle of computations until successive values are almost identi-

cal. It may be necessary to go through 4 or 5 such cycles, but fortunately it usually is not.

4. The calculated final approximations to the dose-response lines are then tested for goodness of fit by χ^2 and for the truth of the assumption of parallelism. Suitable methods are given in the references above and by Finney (1947). If it is fairly obvious from the start that the two separate dose-response lines are not parallel, a modified test should be applied initially in which an independent line is calculated for each substance, and the parallelism test is applied straight away.

5. The log ratio of potency is given by equation 5, p. 19; $\bar{Y}_u$ and $\bar{Y}_s$ are now the mean probits for the unknown and the standard. With equal numbers per group and the same assumed dosage units, the formula does not simplify, as $\bar{X}_s$ and $\bar{X}_u$ are not the same unless the responses are identical in all dosage groups, because of the introduction of the weighting factors mentioned in (2) above.

The variance of M is calculated integrally in performing the operations listed above, but is based on the theoretical variances of the probits and is used with infinite degrees of freedom in determining approximate limits of error.

Fiducial limits of error should be calculated as with other types of assay. The method is the same.

2. Methods of Reducing Error

The error of a quantal assay depends solely on the slope of the dose-response line; assuming that the χ^2 test has shown no significant heterogeneity. It is therefore important to have as steep a line as possible. This may be achieved by selection of the optimal conditions for the test and by selection of as uniform a stock of test objects as possible. Much of the heterogeneity in test objects used in graded response assays can be eliminated as a source of error by appropriate statistical technics such as restrictions in design of tests and the application of covariance analysis. Since this is not yet applicable in practice with quantal responses, more importance must be attached at this stage to securing uniformity in the living material used in tests. It is unfortunate that an early attempt with the use of a pure line of mice in estrogen assays (Emmens, 1939b) did not give a steeper dose-response line than was found with colonies from randomly mated albino mice; but this finding is probably atypical, and the increased use of pure lines in this or other types of assay should be profitable.

Apart from uniformity in genetic constitution, uniformity of nurture and the selection of animals of restricted age and weight is usually found to add significantly to homogeneity in response and to a corresponding

steepening of the dose-response line. It is not uncommon, in quantal assays, to find that low or, less frequently, very high percentage responses tend not to fall on the same line as the rest. Restriction of the range of responses so as to avoid this heterogeneity is necessary when such departures are found, particularly when routine 4-point assays are conducted and there is no check on linearity. Preparation of test animals and the selection of suitable groups with greater uniformity in response has been practiced by some investigators (cf. Palmer, 1937), but care should be taken in procedures of this type not to disturb the log-normal distribution of threshold doses unduly, or the basis of the assay may be invalidated.

3. Reactions in Controls

Particularly when quantal assays involve the death of animals, control groups may show a percentage of non-specific deaths, or even of specific deaths which cannot be eliminated. It has been shown by Finney (1948) that even low percentages of reactions in controls may affect results considerably and that approximate methods for correcting for this may not be successful. The formula for correcting a percentage in a dosage group:

$$p = 100(p_o - p_c)/(100 - p_c),$$

where p_c and p_o are the observed control and experimental percentage reactions, is inadequate without the use of modified weighting coefficients as described by Finney, who gives tables of weighting factors to be used in conjunction with control reactions of up to 40%. The need for such correction is rare in hormone assays.

VI. PREDICTING ASSAY REQUIREMENTS

When a characteristic dose-response line has been established for a particular assay, it may be used in conjunction with the experimentally determined variance, or the theoretical variance in quantal assays, for predicting limits of error and requirements in future assays. Certain assumptions must usually be made, notably that the responses to standard and unknown preparations will not differ much and that the characteristics of the dose-response line will not alter, including the variance of responses.

The minimal error obtainable will occur if the mean responses to the standard and unknown are the same and if equal numbers of responses are obtained to each. The standard error of M is then

$$s_M{}^2 = 2s^2/nb^2$$

where s^2 is the error variance and n is the total number of responses to any one preparation.

The least number of observations required with each substance to attain a certain accuracy is given by

$$n = 2s^2/b^2s_M^2 \qquad (9)$$

where s_M is to be assigned the desired value. The calculated limits in an assay which then turns out to have the same s and b as postulated may not, however, be as narrow as this prediction lays down, unless previous experience is taken into account in making the calculations, and so greater confidence is placed in the value of s and b (particularly the latter) than would otherwise be the case. In many an assay with small numbers of test objects, the fiducial limits of error derived from the internal evidence of that assay alone will much exceed the limits that can be assigned when the slope has been shown to remain constant from time to time and so an average value can be used, based on previous assays and having a smaller standard error. As predictions assume this to be the case, they will be supposed to hold true in the subsequent calculations.

In Table IV, s^2 is 125.9, and b, derived from equation 4a on p. 20, is 24.0 approx. If it is required that s_M shall be 0.04, then $\pm 2ts_M$ is ± 0.08 approx. for $P = 0.95$ as long as n is greater than about 20. Then the limits of error of M are $M \pm 0.08$ as a minimum estimate, or about 83 to 120% of the estimate of relative potency. Such limits will therefore be obtained with $n = 2 \times 125.9/24^2 \times 0.04^2$ observations per substance, whence $n = 252$ approx. This high value for n is not typical of insulin assay by the rabbit method when cross-over technics are used, as the mean square for error is smaller in these more suitably planned assays. A typical value would be $s^2 = 40$ and $b = 45$, whence n would be 25 approx., but since each rabbit is used twice in a cross-over test, only 25 individual rabbits would be needed in all.

In quantal assays, $s^2 = 1$; and an average value for the weighting factor has to be assumed. This is usually taken as 0.5 or a little higher, as 0.5 is the weighting factor for a response of 20% or 80%, and all intermediate responses carry greater weights, up to 0.637 for 50% of reactors. The appropriate equation for n is then:

$$n = 2/wb^2s_M^2 \qquad (10)$$

where w is the weighting factor.

Thus, in a quantal assay with a slope of 5.0 and an assumed w of 0.5, limits of error of 83–120% ($P = 0.95$) can be expected if not less than $2/0.5 \times 5^2 \times 0.04^2$ observations are made per substance, or $n = 100$.

This is a typical value for a test with estrogens by the vaginal smear technic. Various estimates for the numbers of test objects required per substance in various types of assay when limits of error of 80–125% ($P = 0.95$) are desired are shown as examples in Table XII. A complete

TABLE XII

Approximate Number of Animals (not Littermates) Needed per Substance in Assays of Various Hormones Designed to Give Limits of Error 80–125% ($P = 0.95$)

Type of Assay	Reference	No. of animals
Quantitative responses		
Serum gonadotropin, rat ovary weight	B.P.[a] (1948)	13
Adrenal cortical activity, total fermentable sugar in the rat	Eggleston *et al.* (1946)	15
Adrenotropin, chick adrenal weight	Bates *et al.* (1940)	18
Thyrotropin, guinea pig thyroid weight	Rowlands and Parkes (1934)	25
Prolactin, pigeon crop-gland weight	Emmens (1940)	33
Iodoprotein, mouse survival time	Smith *et al.* (1948)	40
Chorionic gonadotropin, rat ovary weight	B.P. (1948)	45
Growth hormone, Hypophysectomized rat weight	Marx *et al.* (1942)	55
Androgens, capon comb growth	Emmens (1939a)	65
Adrenotropin, hypophysectomized rat adrenal weight	Simpson *et al.* (1943)	120
Qualitative responses		
Chorionic gonadotropin, mouse vaginal smear	Emmens (1938)	*ca.* 44
Thyroxine, tadpole leg eruption	Deanesly and Parkes (1945)	48
Estrogens, mouse vaginal smear	Emmens (1939a)	55
Insulin, mouse convulsions	B.P. (1948)	60

[a] British Pharmacopoeia.

assay requires twice this number of animals, as n is calculated for one substance only, either the standard or the unknown. These data were calculated from the information provided in the papers to which reference is made.

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Chapter II

Insulin

By K. L. SMITH

CONTENTS

I. Introduction

Insulin holds a unique position in that it is a potent drug possessing rapid action, that over or under dosage outside very narrow limits is betrayed by very unpleasant symptoms and yet its normal clinical application necessitates daily self-medication by people in all walks of life.

For these reasons it is unfortunate that a chemical test with high inherent accuracy is not available to ensure the uniformity of material issued for use; such a test would be invaluable in determining the conditions necessary to ensure the maximum extraction of this substance, the demand for which is apparently increasing out of proportion to the supply of raw material.

It is fortunate, however, that the qualitative effect of insulin may be readily demonstrated in laboratory animals by the exhibition of a fall in blood sugar, whether indicated by actual blood sugar determination or by the incidence of convulsions relieved by the ingestion of glucose.

II. The Standard Preparation

It is now accepted that assays depending on animal reactions should be conducted so that simultaneous comparison with a standard preparation may be made. The first international standard was established in 1925 and contained by definition 1 unit in 0.125 mg., this unit approximating closely to the clinical unit defined by MacLeod and Orr (1924). Rapidly decreasing stocks and the fact that newer methods of extraction were yielding much purer preparations led to the establishment of the second and present international standard in 1935.

The preparation of this standard and its assay against the previous one is described in the Quarterly Bulletin of the Health Organisation of the League of Nations (1936). It was accepted as having a potency of

22 units/mg. this value being approximately that yielded by the assay on rabbits and somewhat lower than that obtained by the mouse method in all but one of the laboratories.

It is not surprising that the two methods should on this occasion yield different results when it is considered that the response criteria used are not the same and that the comparisons were made between preparations of widely differing purity. Similar differences between the two methods should not occur when preparations having the same degree of purity are being compared.

When required for use the vial may be opened by applying a heated glass rod to a file mark completely encircling it. The contents are then accurately weighed, an operation which must be carried out as rapidly as possible since the preparation, having been dried over phosphorous pentoxide, will take up moisture from the atmosphere. For this purpose very small stoppered weighing bottles may be used, but it has also been found convenient to weigh the opened vial, transfer the contents to the container in which the solution is to be made and then again weigh the empty vial, an operation which with modern air-damped balances may be carried out with sufficient rapidity to avoid any significant uptake of atmospheric moisture.

The weighed standard is dissolved in 0.9% saline acidified with hydrochloric acid to pH 2.5 and containing 0.3% tricresol to prevent the growth of moulds. Sufficient solvent should be taken to produce a solution of suitable potency. It has been found convenient to prepare it to have, at this stage, a potency of 20 units/ml. so that in further dilution to working levels similar operations may be applied to the standard as are employed in preparing dilutions of any test preparation.

If this solution is stored at temperatures near to its freezing point it is considered to be stable for at least a year. The use of this standard for all routine examinations would rapidly deplete the stocks available, and it is therefore customary for each laboratory or group of laboratories to establish its own subsidiary standard the potency of which is accurately related to the international standard. Following their uniform procedure the Reference Standards Committee of the United States Pharmacopoeial Organization have established their own reference standard accurately standardized against the international preparation.

In use it is instructed (U.S.P. XIII) that solution to contain 20 units/ml. be effected in water containing 0.1–0.25% w/v of either phenol or cresol, between 1.4 and 1.8% w/v glycerin and sufficient hydrochloric acid to produce a pH of between 2.5 and 3.5. It is also indicated that such solution should be stored in a cold place protected from freezing and should not be used after 6 months.

III. THE RABBIT METHOD OF ASSAY

1. Design and Interpretation

A. EARLY DESIGNS

A report on the preparation of the first international standard for insulin and the definition of the unit of activity (League of Nations 1926) contained considerations of the principles involved in the assay of this hormone and recorded the test designs which had hitherto been used. The use of the rabbit for the assay had been suggested (Banting *et al.*, 1923) following the discovery that the degree of hypoglycemia produced in rabbits by insulin paralleled the clinical effect in diabetes mellitus.

Since the incidence of hypoglycemic convulsions was usually associated with a blood sugar level of 45 mg./100 ml., the unit of activity had been defined as the smallest amount that would cause this blood sugar level to be reached within 4 hr. of injection. In practice this was interpreted as representing the smallest amount that would cause convulsions within 4 hr. Such units are now replaced by the international unit.

B. THE CROSS-OVER TEST

In comparative assays, completed in one day, the appearance of a particularly sensitive or insensitive rabbit among those receiving either the standard or test preparation must affect the result materially. To overcome this Marks (1925) suggested that the animals used on one day should be tested again in the same groupings, and that on this occasion those which had previously received injections of standard should receive injections of the test preparation and vice versa. He made this suggestion since he had observed that, whereas the level of sensitivity of a colony of rabbits might vary from day to day, the comparative sensitivities of the individuals remained fairly constant.

He suggested that the response of each rabbit be measured as the per cent blood sugar reduction and that an indication of relative activity could be obtained by summing the responses yielded by the rabbits receiving the test preparation and expressing it as a ratio of the corresponding responses to standard. For this purpose he expressed the per cent blood sugar reduction as

$$\% \text{ (Blood sugar) reduction} = 100 \text{ (I.B.S.} - \text{F.B.S.)/I.B.S.}$$

where I.B.S. and F.B.S. are the initial and final blood sugar levels, using for F.B.S. the mean blood sugar level over 5 hr. after injection in samples of blood taken at hourly intervals.

The cross-over test carried out thus could only show whether at the potency assumed the sample was or was not equal in activity to the standard. Many workers (Macleod and Orr, 1926; Culhane, 1929; Herchay and Lacey, 1935) considered that reliance could only be placed on tests which indicated equal activity of the standard and test preparation, and apparently only used indications of departures from this identity to enable suitable adjustments to be made so that it could be reached in subsequent tests.

Marks (1926) considered that the cross-over test, to be efficient, should be capable of demonstrating known differences and showed how by testing known dilutions of standard a curve relating the ratio to true activity could be constructed. In the light of later experience (Marks, 1932) he modified this curve to overcome the correlation exhibited between assumed and estimated potencies and recommended that, to avoid such bias introduced by the use of a curve the slope of which did not strictly apply, an assay should consist of a series of cross-over tests aimed to yield ratios both greater and less than unity.

The protocols of a typical cross-over test have been taken from published data (Fieller, 1940). They are reproduced in Table I and represent the data resulting from a comparison of a test preparation with standard assuming that its potency was 150 units/ml.

Using the appropriate group averages in this case the ratio of test preparation to standard becomes

$$(31.587 + 35.833)/(28.286 + 40.000) = 67.420/68.286 = 0.9873$$

Hemmingsen and Marks (1932) reported from the data available to them that the correlation which existed between the per cent reduction and I.B.S. could be allowed for by reducing the per cent reduction based on a higher I.B.S. by an amount equivalent to 0.22 for each excess milligram per 100 ml.

In the above calculation the combined per cent reduction used for the test preparation (31.587 + 35.833) is based on a value of I.B.S. in excess of that used similarly for the standard (28.286 + 40.000) to an extent

$$(132.429 + 122.333) - (115.571 + 123.833) = 15.358$$

and consequently this per cent reduction should be reduced by an amount

$$0.22 \times 15.358 \text{ or } 3.379$$

The corrected calculated ratio then becomes

$$(67.420 - 3.379)/68.286 = 0.9378$$

The correction suggested by Marks (1932) to refer ratio to estimated potency may now be applied. It consists of increasing the deviation from unity by an amount equal to 0.6 times that deviation.

The true ratio then becomes $0.938 - (0.062 \times 0.6) = 0.938 - 0.037 = 0.901$.

TABLE I

Protocols of a Typical Cross-over Test[a]

Dose	Rabbit	Readings on standard		Readings on test preparation	
		I.B.S.	Response = % reduction	I.B.S.	Response = % reduction
	1	116	40	135	41
Standard	2	128	19	122	26
Day$_1$	3	104	17	135	17
Test preparation	4	121	23	138	36
Day$_2$	5	100	29	131	32
	6	117	26	134	35
	7	123	34	132	36
Sum		809	198	927	223
Mean		115.571	28.286	132.429	31.587
	8	137	41	119	90
Test preparation	9	136	40	108	32
Day$_1$	10	121	42	117	39
Standard	11	113	35	118	38
Day$_2$	12	Convulsion			
	13	123	36	41	41
	14	113	46	45	45
Sum		743	240	734	215
Mean		123.833	40.00	122.333	35.833

[a] Data reproduced from Table 1 (Fieller, 1940).

Thus it would be concluded from this test that the sample possessed a potency 90.1% of that assumed or 135.1 units/ml.

C. THE INTERPRETATION OF A SERIES OF CROSS-OVER TESTS

A review (Fieller *et al.*, 1939a) was made of accumulated data from certain laboratories which had followed Marks' suggestion to assay each sample at assumed potencies so as to yield ratios greater than and less than unity. It demonstrated how such data, whether the relative effect was measured by the ratios of the responses to test preparation and standard or by their differences, could be treated to supply an estimate of the log dose response line (l.d.r.l.) obtaining in the laboratory and to indicate the degree of accuracy to be expected under those conditions.

Fieller (1940) gave an extensive description of the arithmetical procedures involved in this treatment, using response differences as the criterion of relative efficiency and taking as an example a series of seven cross-over tests carried out on one sample at assumed potencies ranging from 100 to 160 units/ml.

The series of cross-over tests taken as an example by Fieller had not been carried out with a view to the subsequent calculations which were enumerated. In a planned assay much computational labor may be saved by arranging that the assumed potencies have equal logarithmic intervals for then we may carry out our main calculations using logs to the base of the dose interval (suitably reduced to yield whole numbers for x) and make suitable correction later.

The test design of the U.S.P. XIII, consists of three cross-over tests with assumed potencies spaced at equal logarithmic intervals, the interpretation of such a test will therefore be considered.

D. THE THREE-ASSUMPTION CROSS-OVER DESIGN

Marks (1936) had suggested that in the course of a cross-over test the animals receiving the test preparation should be split so that they received it at two dose levels, a suggestion which in effect meant the simultaneous conduct of two cross-over tests at two assumed potencies. This design is reflected in the official method for insulin assay adopted by the United States Pharmacopoeial Convention (U.S.P. 1947), which is essentially the procedure described by Lacey (1941) as the method of insulin assay used in the Insulin Committee Laboratory of the University of Toronto and which calls for a comparison with the standard at three assumed potency levels by means of cross-over tests carried out simultaneously. It specifies that the potencies to be assumed should be 89.3%, 100%, and 112% of that labeled.

The response for each rabbit is measured as a per cent reduction

$$\% \text{ reduction} = 100 \,(\text{I.B.S.} - \text{F.B.S.})/\text{I.B.S.}$$

the F.B.S. in this case being the mean level at 1, 3 and a time between 4 and $5\frac{1}{2}$ hr. after injection.

In the official interpretation an index of relative activity is calculated from each cross-over test as

$$\text{Index} = \frac{\text{Mean } \% \text{ reduction effected by test preparation} \times 100}{\text{Mean } \% \text{ reduction effected by standard}}$$

The indexes so obtained are plotted against the assumed potency, the line of best fit drawn and the estimated potency taken as that which corresponds to an index of 100.

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Lacey (1946) describing the interpretation of similar data implied that the indexes should be plotted against log assumed potency and calculated the regression line by the method of least squares. Although the data used by Lacey (1946) was not obtained under the exact conditions specified officially it will be used to show the arithmetical steps needed in the interpretation of a three-assumption cross-over test.

To preserve the similarity of the method with Fieller's treatment of a series of cross-over tests, using response differences, the response will be taken as

$$y = 100 - \text{index}$$

a value suggested by Fieller *et al.* (1939b) and a weight ascribed to each test equal to $w = \dfrac{2n_1 n_2}{n_1 + n_2}$ where n_1 and n_2 are the number of rabbits in each cell of the test.

Lacey (1946) recorded the indexes from three cross-over tests each using 8 rabbits and carried out at assumed potencies of 200%, 100%, and 50% of standard (at 40 units/ml.). If we propose to relate index to log dose, we may since the assumed potencies are equally spaced on the log scale, score whole numbers for the values of $x(= \log$ assumed potency) as given in Table II, making suitable correction later to convert to common logarithms.

TABLE II

Arithmetical Steps in Interpretation of a Three-Assumption Cross-over Test

Test	Assumed potency	w	$x = \log_2$ ^{a} Assumed potency	Index	$y = 100 - \text{Index}$	wx	wx^2	wy	wxy
1	200%	4	+1	67.8	32.2	4	4	128.8	128.8
2	100%	4	0	105.0	−5.0	0	0	−20.0	0
3	50%	4	−1	126.8	−26.8	−4	4	−107.2	107.2
	Sum	12				0	8	1.6	236.0

^{a} Suitably reduced to yield integers:

$$\bar{x} = Swx \div Sw = 0$$
$$Sw(x - \bar{x})^2 = Swx^2 - \bar{x}Swx = 8 - 0 = 8$$
$$\bar{y} = Swy \div Sw = 1.6 \div 12 = 0.1333$$
$$Swx(y - \bar{y}) = Swxy - \bar{y}Swx = 236.0 - 0 = 236.0$$

Then working in logs to base 2 (2 is the ratio between successive assumed potencies).

$$b = \frac{Swx(y - \bar{y})}{Sw(x - \bar{x})^2} = \frac{236}{8} = 29.5$$

and the log activity ratio is

$$M = \bar{x} - \bar{y}/b = \frac{-0.1333}{29.5} = -0.0045$$

Converting to common logarithms

$$M = -0.0045 \times 0.301 = -0.0014 \quad \text{or} \quad \bar{1}.9986 = \log 0.9968$$

Hence the potency is estimated to be 99.7% of that assumed or 39.9 units/ml.

In this case, of course, a similar result would have been obtained if we had taken w as unity, but it may often occur that the constituent cross-over tests are not, by reason of lost responses, exactly balanced. The fuller arithmetical procedure has therefore been included.

In contradistinction to the treatment by differences as described by Fieller, there is no value comparable to s^2 and hence the linearity of the l.d.r.l. cannot be checked nor can the significance of the calculated slope and the limits of error to be attached to the estimate be assessed.

Although the assumed potencies specified for the three cross-over tests in the U.S.P. XIII are spaced equally on the logarithmic scale, the interpretation recorded therein calls for the plotting of the indexes against assumed potency. Perhaps this is because the graphical interpretation which is described in the U.S.P. is rendered even more simple when this procedure is adopted. The difference between results obtained by the plotting of the indexes against assumed potency or log assumed potency is small when the assumed potencies used for the three cross-over tests are as close as specified and when results near to 100% are expected, but would be more important if the design were used with wider dose intervals.

E. THE ESTABLISHMENT OF THE L.D.R.L. FROM MULTIDOSE TESTS

In the designs so far considered the slope of the l.d.r.l. has been determined wholly from changes in response level effected by changes in the injected dose of the test preparation. Test designs in which consideration is given to the parallelism of the l.d.r.l. for the standard and the test preparation have also been described.

Practical difficulties may make it impossible to consider at the same time the linearity of each l.d.r.l., and it may become necessary to establish the relationship of the response to the dose by means of special experiment. Especially is this so if a new criterion of response is being examined.

Bliss and Marks (1939a) described their investigation into the characteristics of the l.d.r.l. for insulin in rabbits when percentage reductions

were used as the response criterion. For this they used data from 8 rabbits, arranged for treatment by means of two randomized 4x4 Latin squares, so that each rabbit received during four testings each of four doses of insulin (equally spaced on a logarithmic scale) each dose being equally represented on each day.

F. A SIX-POINT ASSAY

Bliss and Marks (1939b) also illustrated how this design could be applied to the assay of an unknown sample of insulin against a standard. Their treatment has become a pattern for most of the biological assays in which the response is graded. It has not, however, been applied generally to assay of insulin, solely because of the time needed to collect the data. Under normal conditions, allowing a week to elapse between the separate testings, the time needed to conduct an assay using two doses of standard and two of the test preparation would be 3 weeks.

TABLE III

Protocols from the Assay of Crystalline Insulin 9224B at 22 units/mg.
Ratio between successive doses = 1.667[a]

	Standard			Test preparation			
Rabbit	2 u./ml.	1.2 u./ml.	0.72 u./ml.	2 u./ml.	1.2 u./ml.	0.72 u./ml.	Sums
18	117	116	150	151	158	207	899
23	135	142	146	101	142	191	857
Sums	252 (1)	258 (2)	296 (3)	252 (4)	300 (5)	398 (6)	
64	115	148	163	97	99	160	782
48	134	145	159	137	149	165	889
Sums	249 (6)	293 (1)	322 (2)	234 (3)	248 (4)	325 (5)	
35	159	172	184	155	173	196	1039
8	132	163	147	116	130	157	845
Sums	291 (5)	335 (6)	331 (1)	271 (2)	303 (3)	353 (4)	
46	117	131	135	105	133	157	778
29	169	220	220	138	176	189	1112
Sums	286 (4)	351 (5)	355 (6)	243 (1)	309 (2)	346 (3)	
4	127	144	146	102	123	166	808
21	144	177	240	149	151	178	1039
Sums	271 (3)	321 (4)	386 (5)	251 (6)	274 (1)	344 (2)	
42	133	138	161	139	146	152	869
17	158	188	196	101	142	138	923
Sums	291 (2)	326 (3)	357 (4)	240 (5)	288 (6)	290 (1)	
Total sums	1640	1884	2047	1491	1722	2056	10840

	Days	1	2	3	4	5	6
	Sums	1683	1795	1766	1817	1893	1876

[a] Figures in parentheses indicate day of dosing.

In view of the more recent suggestions that single blood sugar levels without reference to the initial level provide a satisfactory measure of response to insulin (Chapter I, and Young and Romans, 1948), it becomes practicable to apply this design even with the use of three doses each of standard and of the unknown.

To illustrate the interpretation, data shown in Table III have been taken from such an assay using three doses of standard and three of the test preparation, in which the responses were measured as the blood sugar levels (the sum of duplicate readings) at $1\frac{1}{2}$ hr. after the subcutaneous injection of the insulins. The test was completed in 6 consecutive days, injections being made at 9.30 A.M., bleedings made at 11.0 A.M. and the animals being fed uniformly from 12 noon to 4.30 P.M., at which time the uneaten food was removed. The rabbits were allowed access to water during the whole test.

The data was submitted to an analysis of variance, shown in Table IV.

TABLE IV

Analysis of Variance for Data in Table III

Source of variation	Sum of squares	Correction term	Reduced sum of squares	df	Variance
Total	$117^2 + \ldots + 138^2$	$10840^2/72$	61140	71	
Between rabbits	$(899^2 + \ldots + 923^2) \div 6$	$10840^2/72$	21202	11	1927.5
Between days	$(1683^2 + \ldots + 1876^2) \div 12$	$10840^2/72$	2395	5	479
Between doses	$(1640^2 + \ldots + 2056^2) \div 12$	$10840^2/72$	21708	5	4341.6
Residual error			15835	50	$316.7 = s^2$

The significant variance ratio for doses showed that changes in dose were accompanied by real change in response level. A partition of the reduced sum of squares for between doses was made using polynomial coefficients as suggested by Bliss and Marks (1939a) (Table V).

The results of this examination showed that the individual lines relating response to log dose were linear and parallel and that the mean slope differed significantly from zero. Then the log activity ratio may be calculated as

$$M = kID/B$$

where in an assay using three doses of each preparation

$$k = \sqrt{\tfrac{2}{3}} = 1.633$$

and $I = \log_{10}$ of dose interval $= \log 1.667 = 0.2218$, i.e.,

$$M = \frac{(1.6333)(0.2218)(35.6)}{140.3} = 0.0919 = \log 1.236$$

i. Calculation of the Approximate Limits of Error. The limits of error of M are

$$M + ts_m \qquad M - ts_m$$

where $s_m = skI \sqrt{B^2 + D^2}/B^2$. In the example given

$$s_m = \frac{(17.8)(1.633)(0.2218) \sqrt{19683 + 1266.7}}{19683}$$

$$= \frac{933.1533}{19683} = 0.0474$$

and

$$t(P = 0.05) = 2.01$$

The limits of error of $M(P = 0.95)$ are therefore 0.0919 ± 0.0953 $= 0.1872$ and $\bar{1}.9966$ or $\log 1.539$ and 0.9922.

TABLE V

Examination of Dose-Response Relation for Experimental Data in Table III

	St_{100}	St_{60}	St_{36}	T_{100}	T_{60}	T_{36}	$NS(x)^2$	$S(xYp)$	Variance $\dfrac{S^2(xYp)}{NS(x^2)}$	Variance ratio
Difference between samples	-1	-1	-1	$+1$	$+1$	$+1$	72	-302	$1266.7 = D^2$	3.999
Slope of l.d.r.l.	$+1$	0	-1	$+1$	0	-1	48	-972	$19683 = B^2$	62.15
Departure from parallelism	$+1$	0	-1	-1	0	$+1$	48	158	520.1	1.64
Curvature of combined line	$+1$	-2	$+1$	$+1$	-2	$+1$	144	22	3.36	0.01
Opposed curvature of separate lines	-1	$+2$	-1	$+1$	-2	$+1$	144	184	235.11	0.75
Total response in 12 rabbits $= Yp$	1640	1884	2047	1491	1722	2056		Sum	21708.27	
				Residual variance $= 316\ 7$						
					$s = 17.8$					

The true fiducial limits of M are

$$C_pM + t_ps_m \quad \text{and} \quad C_pM - t_ps_m$$

where
$$C_p = B^2/(B^2 - t^2s^2), \text{ and}$$
$$_ps_m = \sqrt{C_p}\, skI \sqrt{B^2 + C_pD^2}/B^2$$

They may also be calculated by applying a simplified formula which is the appropriate modification of that given by Smith *et al.* (1944)

$$C_pM \pm \sqrt{(C_p - 1)(8/3I^2 + C_pM^2)}$$

Using the simplified formula in the example given,

$$C_p(P = 0.05) = 1.0695$$

and the true fiducial limits $(P = 0.95)$ of M are $(1.0695)(0.0919) \pm \sqrt{(0.0695)(0.1312 + 0.0090)} = 0.1970$ and $\overline{1}.9996$, or log 1.574 and log 0.9910.

G. THE TWIN CROSS-OVER TEST

The possibility of designing cross-over tests enabling the slope of the l.d.r.l. for the standard and the test preparation to be compared was referred to by Fieller (1940) and was further examined.

Fieller (1940) made brief mention of cross-over designs using two or three doses of both standard and test preparation which had been carried out and which involved the use of 8 and 18 dosage groups respectively. Ultimately these were replaced by the design which has been referred to as the twin cross-over test (Smith *et al.*, 1944), which is that described in the British Pharmacopoeia 1948, and in Chapter I.

The authors indicated that if a series of twin cross-over tests was carried out on one sample of insulin at the same assumed potency and using the same dose ratio, a condition which is most likely to apply if a sample is being assayed in accordance with the requirements of pharmacopoeias, then providing the separate estimates of s^2 are homogeneous (Bartlett, 1937) the mean activity and its fiducial limits could be calculated by the following method.

For illustration the data from four tests were taken (see Tables VI and VII).

TABLE VI
Summary of Data of a Series of Twin Cross-over Tests
(In each test the assumed strength was 22 units/mg. and *d* 0.3010)

Test No.	$n_1; n_2; n_3; n_4$	$1/w$	$1/w'$	T	U
1	3; 3; 3; 2	1.5000	−0.1667	+ 3.30	28.30
2	3; 3; 3; 3	1.3333	0.0	−15.77	26.10
3	3; 3; 3; 3	1.3333	0.0	− 1.73	44.67
4	3; 3; 3; 3	1.3333	0.0	+ 8.60	46.07

The mean log activity ratio was calculated as

$$M = Xd/Y = (-4.48)(0.301)/106.49$$
$$= -0.0127 = \overline{1}.9873 = \log 0.971$$

The mean activity of the sample was calculated as

$$22 \times 0.971 = 21.4 \text{ u./mg.}$$

The fiducial limits of the mean log activity ratio were calculated as the roots of the equation.

$$X'^2 m^2 - 2(XY)'dm + Y'^2 d^2 = 0$$

where $X'^2 = X^2 - t^2 \bar{s}^2 W = 10701.5$

$(XY)' = XY - t^2 \bar{s}^2 W' = -460.88$

$Y'^2 = Y^2 - t^2 \bar{s}^2 W = -618.51$

whence $(XY)'d = (-460.88)(0.301) = -138.72 = -0.0130 X'^2$

$Y'^2 d^2 = (-618.51)(0.0906) = -56.037 = -0.00524 X'^2$

Thus the quadratic was reduced to the form

$$m^2 + 2(0.0130)m - 0.00524 = 0$$

or

$$(m + 0.0130)^2 = 0.00524 + (0.0130)^2$$
$$= 0.005409 = (0.07354)^2$$

The fiducial limits ($P = 0.95$) of the mean log activity ratio were calculated to be -0.0130 ± 0.0735, i.e., -0.0865 and 0.0605 or log

TABLE VII

Calculations on Data of Table VI and Values of s^2

Test No.	w	wT	wU	w^2/w'	df	S. of sq.	s^2
1	0.6667	+ 2.20	18.87	−0.074	7	142.46	20.35
2	0.7500	−11.83	19.57	0.0	8	303.74	37.97
3	0.7500	− 1.30	33.50	0.0	8	534.65	66.83
4	0.7500	+ 6.45	34.55	0.0	8	650.81	80.35
Sums (1–4)	2.9167	− 4.48	106.49	−0.074	31	1631.66	52.63
	$= W$	$= Y$	$= X$	$= W'$			$= s^2$

$$t^2(P = 0.05, n = 31) = 4.16$$

0.819 and log 1.150, and the fiducial limits of the activity of the sample to be

$$22 \times 0.819 = 18.0 \text{ u./mg.} \quad \text{and} \quad 22 \times 1.15 = 25.3 \text{ u./mg.}$$

A simplified formula was also given which could be applied to those examples in which responses for both days were obtained from all the animals tested or that losses occurred so to render the term $W' = 0$.

This formula necessitates the calculation of the value C_p (Fieller, 1940) which in these instances may be obtained as

$$C_p = X^2/X^2 - t^2 \bar{s}^2 W$$

The fiducial limits of the log activity ratio are then

$$C_p M \pm \sqrt{(C_p - 1)(d^2 + C_p M^2)}$$

H. TRIPLET CROSS-OVER DESIGN

The logical extension of both the twin cross-over design and the three-assumption cross-over test, the triplet cross-over test, has been applied in a collaborative assay of a freeze-dried preparation of globin insulin, undertaken by the Department of Biological Standards, National Institute for Medical Research, and the British Insulin Manufacturers Biological Standardisation Committee (1949).

The layout of this design is shown in Table VIII.

TABLE VIII

Arrangement of the Triplet Cross-over Test

| Dosage group | Treatment in test | |
	Day 1	Day 2
1	Standard (high)	Test (low)
2	Standard (middle)	Test (middle)
3	Standard (low)	Test (high)
4	Test (high)	Standard (low)
5	Test (middle)	Standard (middle)
6	Test (low)	Standard (high)

TABLE IX

Data to Be Extracted from a Triplet Cross-over Test

| Group of rabbits | Mean response to | | Observed mean sum | Observed mean difference $T - St$ | Number of animals |
	Standard	Test			
1	St_3	T_1	Y_1	y_1	n_1
2	St_2	T_2	Y_2	y_2	n_2
3	St_1	T_3	Y_3	y_3	n_3
4	St_1	T_3	Y_4	y_4	n_4
5	St_2	T_2	Y_5	y_5	n_5
6	St_3	T_1	Y_6	y_6	n_6

Since it consists of three cross-over tests carried out at different assumed potencies it could be interpreted by the method described by Fieller (1940).

It can also be interpreted by a method comparable to that for a twin cross-over test, already described. The data to be extracted may be symbolized as in Table IX.

Values of S^2 (mean square between rabbits) and s^2 (mean square within rabbits) are calculated by the methods already considered with regard to the twin cross-over test, both will be determined with $(Sn - 6)$ degrees of freedom.

The important aspects of the assay may be checked by computing the following quantities and their sampling variances (Table X).

TABLE X

Quantity to be calculated		Sampling variance
Agreement between slopes	$(Y_1 + Y_6) - (Y_3 + Y_4)$	$S^2 \left(\dfrac{1}{n_1} + \dfrac{1}{n_6} + \dfrac{1}{n_3} + \dfrac{1}{n_4}\right)$
Departure from linearity	$(Y_1 + Y_3 + Y_4 + Y_6) - 2(Y_2 + Y_5)$	$S^2 \left(\dfrac{1}{n_1} + \dfrac{1}{n_3} + \dfrac{1}{n_4} + \dfrac{1}{n_6} + \dfrac{4}{n_2} + \dfrac{4}{n_5}\right)$
Common slope (U)	$-y_1 + y_3 + y_4 - y_6$	$s^2 \left(\dfrac{1}{n_1} + \dfrac{1}{n_3} + \dfrac{1}{n_4} + \dfrac{1}{n_6}\right)$
Differences in responses (T)	$y_1 + y_2 + y_3 + y_4 + y_5 + y_6$	$s^2 \left(\dfrac{1}{n_1} + \dfrac{1}{n_2} + \dfrac{1}{n_3} + \dfrac{1}{n_4} + \dfrac{1}{n_5} + \dfrac{1}{n_6}\right)$

Working in logs to the base of the *extreme* dose ratio U is an estimate of 4 times the slope of the l.d.r.l. and T is an estimate of 6 times the mean difference between standard and test.

The estimate of $\log_{10}$ activity ratio of the standard and test preparation is

$$M = 2Td/3U$$

where $d = \log_{10}$ of the *extreme* dose ratio.

i. *Calculation of Fiducial Limits.* The calculation of fiducial limits may be made in a similar manner to that recorded for the twin cross-over test.

First the values

$$\frac{1}{w_u} = \frac{1}{n_1} + \frac{1}{n_2} + \frac{1}{n_3} + \frac{1}{n_6}$$

$$\frac{1}{w_t} = \frac{1}{n_1} + \frac{1}{n_2} + \frac{1}{n_3} + \frac{1}{n_4} + \frac{1}{n_5} + \frac{1}{n_6}$$

$$\frac{1}{w'} = -\frac{1}{n_1} + \frac{1}{n_3} + \frac{1}{n_4} - \frac{1}{n_6}$$

are calculated.

Then the fiducial limits are the roots of the equation

$$U'^2 m^2 - (UT)'dm - T'^2 d^2 = 0$$

where $U'^2 = U^2 - t^2 s^2/w_u$

$\quad (UT)' = \frac{2}{3}UT - t^2 s^2/w'$

$\quad\quad T'^2 = \frac{4}{9}(T^2 - t^2 s^2/w_t)$

I. RELATIVE EFFICIENCY OF THE DESIGNS

The most efficient test design based on a given number of responses will be that which yields at the proposed probability level the smallest fiducial range to be attached to the potency estimate extracted from the data.

The formula derived by Fieller (1940) measures the square of the half fiducial range as

$$\frac{t^2 s^2 C_p}{b^2}\left[\frac{1}{n_{St}} + \frac{1}{n_T} + \frac{C_p}{Sw(x-\bar{x})^2}\frac{(\bar{y}_{St}-y_t)^2}{b^2}\right]$$

where

$$C_p = b^2 \Big/ \left[b^2 - \frac{t^2 s^2}{Sw(x-x)^2}\right]$$

and n_{St} and n_T are the number of responses on standard and test preparation. If $\bar{y}_{St} - \bar{y}_T = 0$, and deviations from this cannot be attributed to animal arrangement this reduces to

$$\frac{t^2 s^2 C_p}{b^2}\left(\frac{1}{n_{St}} + \frac{1}{n_T}\right)$$

The portion $\left(\dfrac{1}{n_{St}} + \dfrac{1}{n_T}\right)$ is a minimum when $n_{St} = n_T$, a condition which is imposed by those designs using a cross-over technic or modifications of it. In such cases the expression is reduced to $t^2 s^2 C_p/b^2 N$, where $N = \frac{1}{4}$ the total number of responses.

We have found it convenient to consider as a measure of efficiency the value

$$Wf = \frac{Nb^2}{t^2 s^2 C_p}$$

which may be written $\dfrac{Nb^2}{t^2 s^2} - \dfrac{N}{Sw(x-\bar{x})^2}$, since $\dfrac{1}{C_p}$ may be written

$$\left(\frac{b^2}{s^2} - \frac{t^2}{Sw(x-\bar{x})^2}\right)\Big/ \frac{b^2}{s^2}.$$

If we examine the varying designs which have been discussed keeping the number of responses constant $= 4N$, and considering the extreme log dose interval $= d$ (and equal for both standard and unknown if both are split), we may extract the values shown in Table XI.

TABLE XI

Values of $Sw(x-\bar{x})^2$ and Wf in Various Cross-over Designs Using 4N Responses

Design	Doses of standard	Doses of unknown	$Sw(x-\bar{x})^2$	Wf	df for s^2
Bliss & Marks	2	2	Nd^2	$(Nb^2/t^2 s^2) - (1/d^2)$	$3N-6$
Bliss & Marks	3	3	$2Nd^2/3$	$(Nb^2/t^2 s^2) - (3/2d^2)$	$3N-10$
Twin cross-over	2	2	Nd^2	$(Nb^2/t^2 s^2) - (1/d^2)$	$2N-4$
Triplet cross-over	3	3	$2Nd^2/3$	$(Nb^2/t^2 s^2) - (3/2d^2)$	$2N-6$
3-assumption cross-over	1	3	$Nd^2/6$	$(Nb^2/t^2 s^2) - (6/d^2)$	$2N-6$

From this we would roughly conclude that with a given number of responses the smallest fiducial range is yielded by those designs in which both standard and test preparation are injected at two dose levels, and that the inclusion of an intermediate dose while allowing for a check on linearity to be made widens this range.

It will be appreciated, however, that the apparent difference in efficiency of the designs will be small if $1/d^2$ itself is small compared with Nb^2/t^2s^2.

If we consider the average value of b^2/s^2 encountered in insulin assay on rabbits to be of the order 40, we may construct tables to show the approximate number of responses necessary to yield fiducial limits of given order at the probability levels $P = 0.95$ and $P = 0.99$, when the various designs are used and the extreme dose ratios are those described in the official tests of the B.P. 1948 and the U.S.P. XIII (Table XII).

TABLE XII

The Approximate Number of Rabbit Responses Needed to Yield Fiducial Limits of Given Order When $b^2/s^2 = 40$

Design	Doses of standard unknown		Error Wf	10% 583.22		15% 271.26		25% 106.50		50% 32.25	
				$P = 0.99$	$P = 0.95$	$P = 0.99$	$P = 0.95$	$P = 0.99$	$P = 0.95$	$P = 0.99$	$P = 0.95$
Bliss & Marks	2	2		404	233	196	112	84	49	34	22
Bliss & Marks	3	3		407	235	200	115	88	51	38	25
Twin cross-over	2	2	$d = 0.3010$	404	233	197	114	85	51	35	23
Triplet cross-over	3	3		407	235	201	116	89	53	40	26
3-assumption cross-over	1	3		441	255	233	135	123	72	73	44
Bliss & Marks	2	2		466	270	255	147	146	84	96	55
Bliss & Marks	3	3		502	284	297	167	251	104	131	76
Twin cross-over	2	2	$d = 0.0983$	466	270	255	149	148	186	97	58
Triplet cross-over	3	3		502	289	297	169	256	106	133	78
3-assumption cross-over	1	3		818	462	606	343	494	279	444	251

The conclusions to be drawn from this table are that when $b^2/s^2 = 40$ all designs are roughly of equal efficiency when high orders of accuracy are aimed for and the dose ratio is reasonably wide. The slight loss of efficiency, encountered with the three-assumption cross-over design in comparison with other designs when $d = 0.301$, is greatly increased if

d = 0.0983, which is the extreme logarithmic interval specified in the U.S.P. XIII.

J. TEST FOR DELAYED ACTIVITY

Although the clinical action of the insulin preparations which possess delayed activity may not be reflected identically in the response of the normal unfed rabbit, a test for delayed action may be performed on rabbits which gives some indications of the relative efficiency of such preparations and may be used to examine them for uniformity either by
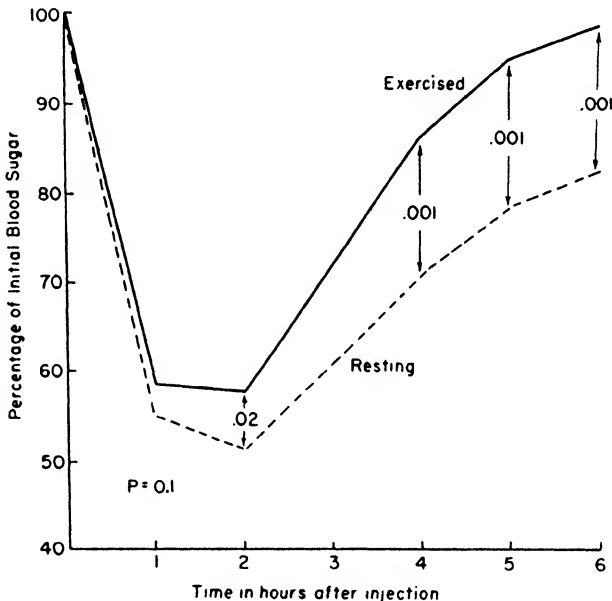

FIG. 1. Graph to show the reliability of the differences between the responses of "exercised" and "resting" rabbits after injection of soluble insulin. The significance of the differences at each hour is shown by the arrows. (From Thorp, 1944a.)

making comparison with soluble insulin or preferably with a standard preparation of like composition.

In such tests the cross-over principle is employed. The data recorded are the mean blood sugar levels of the groups of rabbits at fixed intervals after injection, and may or may not be expressed as a percentage of the initial level. There seems some advantage in expressing the level in this way since if it is then plotted against time, the resulting curves will have a common origin.

It is customary to carry out the comparison at one assumed level only, and since the character of the delay acting insulins may be other-

wise impaired the injection is made without dilution and at equal levels of concentrations for both standard and test preparation. For this reason the volumes to be injected will be very small and of the order 0.01 ml. or 0.02 ml. for preparations containing 80 or 40 units/ml. Injections of such volumes may be readily made by means of the micrometer syringe designed by Trevan, and Lacey (1946) has also referred to the use of 0.25-ml. syringes graduated in 0.01 ml. for this purpose.

The precision of the U.S.P. XIII test for delayed activity has been examined by Bliss (1949); he considers the test can distinguish finer differences than it is called to make in practice.

Thorp (1944a) has shown that the handling of the rabbits during the course of the assay affects considerably the speed with which their blood sugars return to normal. His results shown in Fig. 1 indicate that this is more marked in the case of soluble insulin than in the case of delayed-acting preparations.

2. Manipulative Procedures

A. RABBIT COLONY

If it were possible to set aside sufficient space for breeding or to purchase such animals, the use of one breed of rabbits for insulin assay might be preferable.

Bliss and Marks (1939b) considered the ways in which the reactions of individual rabbits could affect the accuracy of the assay. They concluded in their experiment that those of the Sandy Lop Breed were less sensitive to insulin than the 12 Himalayans which were retained and on which their subsequent calculations were made. A valid treatment in which individual sensitivities are allowed for will be referred to later, its use here might have made all animals in this group equally suitable.

Among the Himalayans retained by Bliss and Marks, however, there was no evidence that the rabbits varied in their reactions either to dose changes or to differences in days although there was still evidence of a wide variation in their overall susceptibility. Certain rabbits showed more erratic responses than the others, and it was suggested that it might be profitable to replace such animals in future tests. Retaining the Himalayans the estimate of variance was $s^2 = 41.4$ ($77df$), but if the erratic rabbits were removed this was reduced to 28.7 ($49df$).

In the conduct of cross-over tests variations in the slopes of the l.d.r.l. of individual rabbits would produce larger effects on the value of s^2 when the doses administered to the rabbit were more widely spaced. Fieller (1940) noted that in the simple cross-over tests he had examined and which had been carried out on mixed breeds there was no evidence of this. From similar tests carried out from 1933–1938 a mean estimate

of $s^2 = 35.18$ (5825*df*) was recorded (Fieller *et al.*, 1939b). Data from the same laboratory for the period 1941–1945 when larger differences in the doses were effected by use of the twin cross-over design the estimate of variance was 48.16 (3749*df*).

This higher variance could indicate that variations in the slopes of l.d.r.l. for individual rabbits did occur but such an assumption could not be substantiated, nor could it be assumed that use of rabbits of one breed would have obviated this. (In all the laboratories with which we are acquainted, the colonies used for insulin assay are composed of mixed breeds purchased from reputable dealers and weigh from 2–3 kg.)

We find that often on arrival the rabbits' ears are infested with mites, a condition which may be successfully treated with phenolized oil, the use of which as a prophylactic for this purpose is also to be recommended.

B. SELECTION FOR TEST

When animals are being selected for test the record cards of those considered suitable may be shuffled and dealt into heaps, corresponding in number to the number of dosage groups, until the number decided as convenient for use has been selected.

The doses to be assigned to these groups are decided by further randomization. In 2-day tests in which the dose on the second day is determined by that administered on the first day, this may be done by labeling the heaps A, B, C, etc., and writing these letters on small cards, preparing other small cards to cover the dosage groups and withdrawing these cards from a box at random.

In 4- or 6-day tests a more elaborate randomization may be employed such as is described by Bliss and Marks (1939a) using the basic patterns tabulated by Fisher and Yates (1938).

The basic pattern to be used is first selected at random. The pattern must then be further randomized by interchanging rows and columns by use of shuffled numbered tags or by use of random numbers. The doses are assigned to the letters and finally the groups of rabbits or individual rabbits assigned to the rows.

Since this procedure is laborious we considered it worth while to record all the 4x4 combinations possible, paying due regard to the relative frequency to be applied to the basic patterns, and to assign a consecutive number by randomization to each combination, and we are now able to make our final selection by a method similar to that used above to select the basic pattern.

Although theoretically similar frequencies in the 6x6 design should be considered, in practice it is adequate to make randomization of the one block given by the interchange of rows and columns.

It is, we believe, normal practice on the completion of a test to split up the groups of animals used so that random selections for a new test may be made. In the conduct of cross-over tests we have made use of a modification in which, on the day a test is completed, further groups of animals are injected with dilutions of a new test preparation and these groups together with the original groups receiving standard form the beginning of a new test. The economy in animals is considerable, and the procedure has only the objection that the groups retain their identity for three consecutive appearances, losing it when they have received the test preparation on the day the second test is completed.

C. COLONY DIET

We do not consider that the nature of the colony diet, providing it is adequate, has marked effect on the accuracy of insulin assay. In practice we find that feeding a mixture of bran and oats supplemented with fresh green food and allowing access to water continuously gives results roughly parallel to those obtaining in other laboratories. We understand too that in another laboratory the use of cubed diet without supplements of fresh green food (Bruce and Parkes, 1946) has proved equally satisfactory.

D. DOSAGE OF ANIMALS

In single-day tests it would be imperative that the doses be administered on a strictly defined basis, e.g., units/kg., and as has been previously noted the presence of an unduly sensitive or insensitive rabbit would materially affect the result obtained.

Use of the cross-over technic or modifications of it allows for animals of widely differing sensitivities to be used without bias, but even so it is wise to avoid using those animals which respond only slightly to the defined dose or to an extent approaching convulsive levels. This could be achieved by submitting all animals to a prior standardization and discarding those which respond outside certain predetermined levels.

Marks (1925) considered that though doses could be related to the weight of the animal the dose received by an animal throughout a test should be constant and not fluctuate with its weight changes, which would of course be small if they occurred at all. This principle is also implied in the conditions specified in the U.S.P. XIII.

Constancy of dose in a test is also maintained in a practice which we have followed to render a greater proportion of the colony available for test. In this method that dose which will produce a satisfactory response is determined for each animal and is expressed in milliliters of a standard solution containing 2 units/ml. This volume is called the "standard

volume" and may vary from 0.3 to 0.8 mils. Whenever an animal appears on test it receives its "standard volume" of the preparation suitably diluted to allow for different levels of dosage to be employed. This procedure has been examined critically (Fieller *et al.*, 1939b) and shown to be valid.

We may, at this point, consider what constitutes a suitable response. Published data (Bliss and Marks, 1939a; Fieller *et al.*, 1939a, b) indicate that the change in per cent reduction induced by unit change in log dose is of the order of 40. If in split tests a dose ratio of 2 to 1 ($d = 0.301$) is employed the change induced in response by changes from high to low dose will be 12. If, therefore, a response, in terms of per cent reduction of 30–40 is aimed for on the "standard dose," responses to lower doses would be 18–28 which are not too low.

It has been customary to make the injection of insulin in concentrations of the order 2 units/ml. using 1-ml. tuberculin syringes. These are usually of high order of accuracy, but it will be appreciated that if the method of dosage employing use of "standard volumes" is followed inaccuracy in graduation of the syringe except of an erratic type, providing it is used without change for the whole test, will be of no importance.

Lacey (1946) has reported that satisfactory results may be achieved by making injections without dilution by means of micrometer syringe or by using a 0.25-ml. syringe graduated to 0.01 ml. This it is claimed does not mask the difference in action between insulins of differing purity which has greater importance in experimental work than in the routine assay of insulin solutions for clinical use.

It has also been customary to make the injections subcutaneously. Young and Romans (1948) have reported experiments which show that intravenous injections, with blood sugar levels determined at 50 min. after injection, are perfectly satisfactory. Our own experience is that the levels of dosage required for intravenous or subcutaneous injection are the same and that one route has little advantage over the other from the point of view of accuracy, although the subcutaneous injection has the advantage of manipulative simplicity.

E. BLOOD SAMPLES

The method adopted for the taking of blood samples will depend on individual preferences; the following procedure has been used quite satisfactorily over a long period.

The blood is removed via the ear by venipuncture. The ear of the rabbit is first shaved, a small paper clip is fastened to the base, and the veins are further dilated by means of a 32 candlepower carbon filament lamp which serves also to illuminate the venous pattern of the ear. An

incision is made in the external vein and in the first instances as near to the base of the ear as is convenient. Incision of the larger mid-veins soon renders the ear unsuitable for the taking of further samples. The blood is encouraged to flow by the minimum of massage and is collected

Fig. 2. The restraining box for bleeding rabbits.

into a small pot containing a few crystals of potassium oxalate, from which it is pipetted into suitable deproteinizing solutions. Subsequent bleedings are induced at this puncture by the same operations and usually, but not always, necessitate reopening the puncture by means of the needle. The necessary dilation of the veins may be accomplished by the use of xylol, which does, however, tend to harden the ear.

Although the use of heparin or special resins will suggest themselves for the purpose of rendering shed blood non-coagulable, the use of potassium oxalate as described has been found quite satisfactory. If more accurate oxalation is desired it may be obtained by pipetting exact volumes of potassium oxalate solution into clean tubes and then drying off in an oven.

It is to be expected that excitement, with the consequent liberation of adrenalin, will cause a considerable rise in blood sugar level, and it is
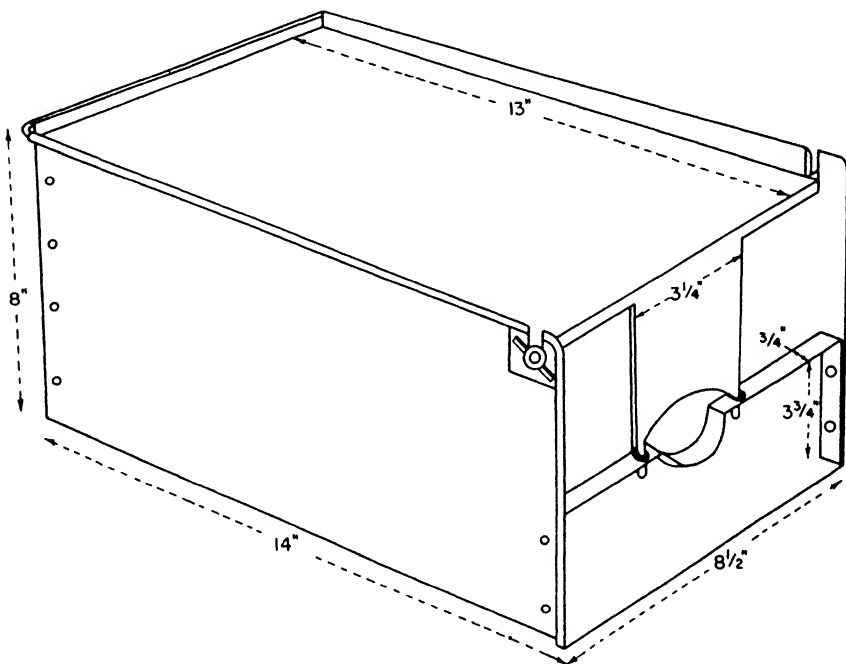
FIG. 3. Details of the rabbit box. (From Thorp, 1944b.)

therefore desirable that a minimum amount of disturbance to the animals should be allowed. We have been content to allow the animals freedom of movement between bleedings by housing them in suitable cages whence they are removed in turn to a restraining box so that the bleeding may be more easily accomplished (Fig. 2). The front of this box is in the form of stocks secured by means of a sliding lid, the box is also fitted with a movable partition so that rabbits of varying sizes may be accommodated.

This removal from the cage to box cannot be done without some disturbance, and the effect of the treatment on the blood sugar level of untreated fasting animals has been studied. It was concluded that removing the animals for the taking of blood samples at hourly intervals

would not affect the response unduly, especially when it was considered that each animal would receive equivalent treatment.

Another procedure consists of restraining the animals for the duration of the test either by tying the animals in a bundle by means of a cloth square so that only the head is visible, a condition which is endured without struggle, or by confining them to the restraining box. For this purpose Thorp (1944b) found a specially designed metal box reproduced diagrammatically in Fig. 3 to be most convenient. It had the advantage that it was readily cleaned and so designed that damage to the spine was practically impossible. Thorp found that the animals soon accustomed themselves to remaining in such boxes for long periods without struggling. It is our experience that struggling does occasionally occur; the disturbance then is more severe than that caused by removal to a separate bleeding box and is of course less uniform for the group of animals.

Thorp (1944a) compared the effect of this procedure with that in which a uniform amount of exercise was applied to each rabbit before each bleeding and showed that the curves relating blood sugar to time differed markedly between the exercised and non-exercised rabbits when treated with soluble insulin. The graph prepared by him is reproduced in Fig. 1.

F. BLOOD SUGAR DETERMINATIONS

The method of Hagedorn and Jensen (1923) using Somogyi's (1930) method of protein precipitation lends itself most readily to multiblood sugar analyses and relatively unskilled workers soon attain a high degree of accuracy in its use.

We rely on the collection of 1 ml. of blood for individual determinations and for pooled values over 5 hr. 0.2 ml. is taken at each hour and pipetted into the same deproteinizing tube.

Thus 1 ml. of oxalated blood in 8 ml. of acid-zinc sulfate solution (12.5 g. zinc sulfate and 31.25 ml. N sulfuric acid per liter) is deproteinized by the addition of 1 ml. $0.75N$ sodium hydroxide. Vigorous shaking and subsequent filtration through a 9-cm. Whatman No. 1 filter yields sufficient filtrate, representing a 1:10 dilution of blood, to allow duplicate determinations using 2 ml. filtrate (= 0.2 ml. blood) to be made.

Two milliliters of filtrate are pipetted into 2 ml. of alkaline potassium ferricyanide solution (1.65 g. potassium ferricyanide and 10.6 g. anhydrous sodium carbonate per liter) contained in a boiling tube 6 in. by 1 in. and transferred to a boiling water bath for 15 min. After cooling, 3 ml. of zinc iodide solution (potassium iodide 2.5 g., zinc sulfate 50 g., sodium chloride 250 g. per liter) and 2 ml. of dilute acetic acid (3% glacial acetic acid) are added and the liberated iodine is titrated using $0.005N$

sodium thiosulfate. The difference in the titer needed for the sample tube and for a tube (the blank) prepared and treated similarly using 2 ml. of distilled water is proportional to the quantity of glucose present in the filtrate. This titer difference representing glucose concentration would of course serve for any calculations to be applied in an insulin assay, but it is customary to express it in milligrams glucose/100 ml. of blood.

Whether to contribute to greater accuracy or to avoid lost readings through accident, it is customary to determine blood sugars in duplicate. Actually our practice has been to make the bleedings in duplicate and make one determination on each filtrate. From data available to him and which had been obtained in this manner but using the Shaffer and Hartman (1921) method of blood sugar determination, Fieller (1940) considered the standard error of a single determination to be of the order 3.7 mg. and calculated that with an I.B.S. of 120 mg./100 ml. and F.B.S. ranging from 70–100, values ranging from 6.4–8.1 would be contributed to the residual variance of a percentage reduction. If only single determinations were made we would expect the residual variance to be increased by a further amount ranging from 6.4–8.1 under similar conditions.

Since the efficiency of an assay is inversely proportional to the residual variance of a single response but proportional to the number of responses obtained, any loss of efficiency resulting from the use of single in place of duplicate determinations could be allowed for, in the extreme case quoted above, by multiplying the number of responses by $(s^2 + 8.1)/s^2$, or $1 + 8.1/s^2$.

If we expect s^2 to be of the order 36 (Fieller, 1940) this factor would then be 1.225.

G. CRITERION OF RESPONSE

Both the U.S.P. XIII and the B.P. 1948 preserve the early conception that the most informative response is the blood sugar level measured several times over 5 hr. after injection and related to the I.B.S.

Whereas the B.P. 1948 does not specify the nature of the relationship of F.B.S. to I.B.S., the U.S.P. XIII instructs that a per cent reduction be calculated, and indicates that the indexes, obtained by relating the mean per cent reduction effected by the test preparation to the mean per cent reduction effected by the standard, are linearly related to the dose levels.

It has also been shown (Bliss and Marks, 1939a; Fieller, 1940) that a linear relationship exists between the per cent reduction and the log dose. Many workers (Hemmingsen and Marks, 1932; Fieller, 1940;

Bliss and Marks, 1939a) however, have shown that if the F.B.S. is related to the I.B.S. in this manner a correlation still exists between the resulting per cent reduction and the I.B.S., and methods have been described by which this degree of correlation may be assessed and corrected.

Since it was found in one laboratory that the correlation factor had remained stable over a number of years it was suggested that adjusted responses should replace the per cent reductions and for this purpose per cent reductions − 0.3 I.B.S. (in mg./100 ml.) were used.

More recently it has been suggested (Emmens, 1948; Young and Romans, 1948) that an efficient response is the blood sugar level determined at that time at which experience suggests the lowest level is reached. Thus with intravenous injections Young and Romans were content to take single blood samples after 50 min. had elapsed; Pugsley and Rampton (1948) have confirmed that the use of the single reading following intravenous injections, as a measure of response, compares very favorably with that of the mean blood sugar level over 5 hr. related to I.B.S. following subcutaneous injections. Data presented in this chapter to illustrate the interpretation of Bliss and Marks show that with subcutaneous injection blood sugars at $1\frac{1}{2}$ hr. yield a satisfactory response.

Having established the response the question arises how best it may be used to interpret the potency of the test preparation. Two methods are available, one in which response differences are considered and the other in which treatment is by ratios.

Fieller (1940) considered that the only advantage of one over the other was that differences were more amenable to statistical analysis. Methods in which ratios are used (the mean ratios of mean per cent reduction of groups) do not allow a value comparable to s^2 to be calculated, and hence assumptions of linearity cannot be checked and fiducial limits cannot be calculated; presumably if the individual rabbit ratios are used they could be treated to form an estimate of s^2, but the index would then be the mean of the individual ratios and not the ratio of the mean responses, which would produce yet another criterion.

If a laboratory is ever to employ the Bliss and Marks design, it would appear that an interpretation of response equally applicable to that and to the cross-over design should be adopted if only for the sake of uniformity.

H. FASTING PERIOD AND FREQUENCY WITH WHICH ANIMALS MAY BE USED

The frequency with which animals may be used will depend to a large extent on the fasting period imposed and the bleeding schedule.

A 6-day interval between usage will allow almost any accepted fasting period and any bleeding schedule to be applied.

With this interval it has been found convenient to arrange that the animals are not fed on the day before test nor on the day of the test until the last bleeding, 5 hr. after injection has been made. On occasions, to accommodate the usage of animals twice in one week, the animals have been fed in the morning of the day before test and the remaining food is removed at 4.30 P.M. A similar pretesting treatment has been imposed for the completion of the test with a 2-day interval between the two halves of the test.

No great differences in reaction were noted on these occasions except that it was found necessary to increase the dose of insulin injected, by approximately 30%.

If the bleeding schedule is such that only one blood sample is taken (Young and Romans, 1948), the frequency of use may be greatly increased. The data quoted in Table IV were obtained on 6 successive days by taking blood samples $1\frac{1}{2}$ hr. after the subcutaneous injection of insulin and arranging that the animals were fed uniformly from noon to 4.30 P.M.

It is not considered that this treatment is too severe; in fact animals used in this way are fed more uniformly, and the number of bleedings per rabbit in 1 week is less than the number carried out in 1 day if the method followed is that which has hitherto been considered normal. Young and Romans reported that with a week's rest between successive tests it was possible to use each animal in this manner for 4 to 5 tests.

IV. THE MOUSE METHOD OF ASSAY

1. Design and Interpretation

A. EARLY DESIGNS

The fact, that mice, though able to withstand the effects of large doses of insulin at normal environmental temperatures, showed characteristic convulsive symptoms at elevated temperatures, led to their extensive use.

The dose needed to cause 50% of convulsions, the mouse dose of insulin, was estimated by Hemmingsen and Krogh (1926) under their conditions to have $\frac{1}{800}$ the value of the original Toronto rabbit unit. It was reported purely for the sake of interest since the inadequacy of this measure of response was appreciated.

Construction of dose-response curves resulted sooner with this response than had been the case with the rabbit response, although as

first described some differences in the nature of the relationship of the percentage of convulsions to dose were recorded. Hemmingsen and Krogh (1926) presented graphs in which the percentage of convulsions was linearly related to log dose. Trevan and Boock (1926) plotted per cent convulsions against dose, but this was done for expediency in calculation since they considered the ratio of the doses necessary to induce identical changes in response level on different days to be equal. A uniform treatment of data supplied by animals yielding "all or none responses" was suggested by Gaddum (1933) which indicated that a mathematical function of the per cent response, its normal equivalent deviation (N.E.D.) was linearly related to log dose. Hemmingsen (1933) applied this treatment specifically to insulin assays using data from his own laboratory and that published by others (Trevan and Boock, 1926; Trevan, 1927) and concluded that not only was the N.E.D. of the per cent response of mice to insulin linear to log dose but that the variation in the slopes of the l.d.r.l. so calculated exceeded that expected by the random sampling of mice.

B. THE 2x2 TEST

Tests using two doses of standard and two doses of the test preparation at the same time enable checks to be made on the parallelism of the slopes of the l.d.r.l. for standard and test preparation.

Table XIII shows the responses which were obtained in the course of such an assay and records also the transformation to N.E.D., the weighting factor appropriate to it (= B), and the overall weighting factor to be attached to the points (= nB).

<p style="text-align:center">TABLE XIII</p>

Dose	Standard high	Standard low	Test high	Test low
Response	18/24	1/24	15/24	4/24
N.E.D.	0.634	−1.731	0.3186	−0.9674
B	0.539	0.199	0.613	0.45
n	24	24	24	24
nB	13.44	4.8	14.64	10.8

<p style="text-align:center">Ratio of high to low dose 100/60 = 1.667.</p>

The calculations to be applied to the responses to standard are shown in Table XIV, and similar treatment of the responses to the test preparation will also be necessary. Since the dose ratios for the standard and the test preparation are equal, the calculations may again be simplified by using integers for x = log dose, writing 1 for log high dose and 0 for

log low dose and making suitable correction later to convert to terms of common logarithms.

TABLE XIV

Dose	Response	N.E.D. = y	$nB = N$	x	Nx	Nx^2	Ny	Nxy
60	1/24	−1.731	4.8	0	0	0	−8.3088	0
100	18/24	0.634	13.44	1	13.44	13.44	9.0586	9.0586
Sum			18.24		13.44	13.44	0.7498	9.0586

$$\bar{x} = SNx \div SN \qquad 13.44 \div 18.24 = 0.7368$$
$$\bar{y} = SNy \div SN \qquad 0.7498 \div 18.24 = 0.0411$$
$$SN(x - \bar{x})^2 = SNx^2 - \bar{x}SNx \qquad 13.44 - 9.9032 = 3.5368 = p$$
$$SNx(y - \bar{y}) = SNxy - \bar{x}SNy \qquad 9.0586 - 0.5525 = 8.5061 = q$$
$$[SNx(y - \bar{y})]^2 \div SN(x - \bar{x})^2 \qquad (8.5061)^2 \div 3.5368 = 20.4574$$
$$1/SN \qquad 1 \div 18.24 = 0.0548$$

These values together with those obtained by similar treatment of the data for the test preparation are collected together in Table XV where the additions and subtractions subsequently called for may be

TABLE XV

	$1/SN$	q	p	q^2/p	$\bar{y}$	$\bar{x}$
Standard	0.0548	8.5061	3.5368•	20.4574	0.0411	0.7368
Test	0.0393	7.9926	6.2151	10.2785	−0.2269	0.5755
Sum	0.0941	16.4987 = Q	9.7519 = P	30.7359		
Difference					0.2680	0.1613

$$b = Q/P = 1.692 \qquad Q^2/P \qquad 27.920$$
$$Sq^2/p - Q^2/p \qquad (\chi^2 \text{ slopes}) \qquad 2.8159$$

conveniently made. Working in common logarithms the log activity ratio of unknown to standard is

$$M = d[(\bar{x}_{St} - \bar{x}_T) - (\bar{y}_{St} - \bar{y}_T)/b]$$

where d = log dose ratio which in the example is log 1.667 = 0.2218 thus $M = 0.2218[0.1613 - (0.2680/1.692)] = 0.0006 = \log 1.001$

We therefore estimate the potency of the unknown to be 100.1% of that assumed. The rest of the calculations follow as indicated in Chapter I. It may happen that zero or total convulsions are encountered in a test. When using groups of 24 mice the convention of treating such responses as $\frac{1}{2}$ or $23\frac{1}{2}$ out of 24 has been adopted by the author. If in any test two total or two zero responses have been recorded, these

responses have only been used to estimate the slope of the l.d.r.l., and the estimate of relative potency has been based on the remaining two responses.

Such treatment may not be perfectly valid, but it seems preferable to the discarding of any data which indicates the relative potency of the standard and test preparation.

Although simplified calculations have been suggested for the interpretation of 2x2 assays, the more extensive method described is preferable. If certain conditions are standardized, much computational labor may be saved by the preparation of suitable tables.

C. CROSS-OVER TESTS WITH MICE

Hemmingsen (1939) has described the application of the cross-over technic to the mouse method of assay. A unit cross-over test using a single dose each of standard and test preparation was conducted so that on the second day those mice which had previously received the standard now received the test preparation and vice versa. Hemmingsen imposed a further restriction in that the group was preserved intact and used for a series of cross-over tests at varying assumed potencies. Hemmingsen interpreted the data simply by recording the difference in the proportion of mice convulsing on standard and test preparation in each test and relating this difference to the l.d.r.l. calculated by dividing the range of the differences effected by extreme change in assumed potency by the log of the ratio of these assumed potencies. He pointed out that providing the convulsion rates lay between 10% and 90% this result would be practically the same as would have been obtained if probits had been used.

Hemmingsen assessed accuracy by recording the standard deviation of the estimated potencies and concluded that by making the comparison between standard and test preparation in a series of cross-over tests on the same mice the standard deviation of a test comprising 160 mice was reduced from 12–25% to 7–10%.

2. *Manipulative Procedures*

A. ANIMAL COLONY

The assay of insulin in mice has been successfully applied using both animals purchased from dealers and those bred within the laboratory. The latter course has the advantages that the animals are likely to be more uniform and that they are not subjected to the disturbances associated with the delivery journey and with the change in diet. We have attempted to overcome the effects of these disturbances by allowing an interval of at least 7 days to elapse between the receipt of the animals

and their use for test purposes. It has been our practice to house the animals on sawdust in sheet metal boxes with a mesh lid, in a room having a temperature range of 65–70°F. These boxes have dimensions 12 in. by 12 in. by 6 in. and are considered adequate to hold 35 mice weighing up to 30 g.

B. COLONY DIET

Recent work (Rowlinson and Lesford, 1948) has suggested that a change in colony diet can cause a change in the slope of the l.d.r.l. and hence, of course, affect the overall efficiency of the test. The data indicated that with a diet consisting of bread, the mean slope of the l.d.r.l. was 4.68, with a mixed diet fed in the form of cubes and containing more protein and fat but less carbohydrate, the slope of the l.d.r.l. was 3.96. The difference between these two values was stated to be significant at a $P = 0.95$ level. With both diets the animals received water *ad libitum*.

We have examined the slope of the l.d.r.l. over two periods whilst mixed diets were being used. In the period November, 1944, to July, 1945 covering 269 tests, the mean slope was estimated to be 5.4382, though significant heterogeneity was indicated by the value of $\chi^2 = 407$. During the first half of 1948 the mean slope from 231 tests was estimated to be 5.3206; in this case no significant heterogeneity was indicated by the observed value of $\chi^2 = 284$.

C. FASTING PERIODS

It is possible that the use of some precise fasting period would provide more uniform animals for test purposes. A convenient procedure, however, has been followed by us for several years, in which those animals set aside for insulin assay are fed each day at noon. On the next day all are considered equally fasted and suitable for test. Those required are removed to clean boxes and subjected to test from 1 to 6 hr. later.

TABLE XVI
The Influence of Extended Period of Fasting on $C.D._{50}$ and Slope of l.d.r.l.

Time tested	No. of tests	log $C.D._{50}$ in mu./g. and S.E.	χ^2 between tests	Slope of l.d.r.l. and S.E.	χ^2 between tests
Morning	97	$\bar{1}.7409\ (0.011)$	769.9	5.470 (0.143)	6.43
Afternoon	134	$\bar{1}.7407\ (0.009)$	870.7	5.193 (0.116)	155.95

	χ^2 between times	0.00		χ^2 between times	2.27
Critical $\chi^2 P = 0.05$	$n = 133$		$n = 96$	$n = 1$	
	166.35		124.50	3.84	

The influence of this difference in the fasting period on the $C.D._{50}$ (the dose causing 50% of convulsions) and on the slope of the l.d.r.l. during 231 tests has been examined by comparing those tests carried out during the morning with those carried out during the afternoon. The summarized results of this examination are shown in Table XVI.

They indicate that the extended fasting period imposed by delaying the tests until the afternoon has no effect on the sensitivity level of the mice and no significant effect on the slope of the l.d.r.l.

D. SELECTION OF ANIMALS FOR TEST

i. *Selection on Basis of Weight.* The fasted mice are weighed and sorted into groups with restricted weight ranges. Hemmingsen (1939) has reported the use of mice weighing 12 g. but it is our practice to limit our use to those weighing 17–30 g., segregating them into groups weighing 17/20 g., 20/25 g., 25/30 g. The mice for any one test are taken from one only of these weight groups, and for the purpose of this test are considered to be of equal weight. The influence of different weight groupings on the $C.D._{50}$ and on the slope of the l.d.r.l. during 231 tests has been examined.

The summarized results of this examination are shown in Table XVII.

TABLE XVII

Influence of Weight Groups on the $C.D._{50}$ and on the Slope of the l.d.r.l.

Weight group	No. of tests	log. $C.D._{50}$ in mu./g. and S.E.	χ^2 between tests	Slope of l.d.r.l. and S.E.	χ^2 between tests
17/20 g.	80	$\bar{1}.7040$ (0.012)	535.8	5.156 (0.151)	101.88
20/25 g.	123	$\bar{1}.7604$ (0.009)	868.3	5.385 (0.125)	125.46
25/30 g.	28	$\bar{1}.7518$ (0.016)	129.9	5.396 (0.259)	35.79

	χ^2 between groups 14.7			χ^2 between groups 1.51	
Critical $\chi^2 P = 0.05$	$n = 122$	$n = 79$	$n = 27$	$n = 2$	
	154.0	104.98	40.113	5.99	

They indicate that whereas in the case of the 20/25 g. and 25/30 g. groups dosing may be based on the mean of the weight range, in the case of the 17/20 g. some extra compensation is called for to allow for their greater sensitivity. The different weight groupings have no effect on the slope of the l.d.r.l.

ii. *Selection on Basis of Previous Usage.* In our practice the mice surviving the test are used again, and those chosen for test are segregated according to whether they are new mice, mice which have been used once, used twice, etc.

The effect of previous usage of mice on their reactions to insulin during 231 tests has been examined. The summarized results of this

examination are shown in Table XVIII. They show that the prior usage of mice has a significant effect on the $C.D._{50}$ which is lower for new mice and those used once.

TABLE XVIII

Influence of Previous Usage on the $C.D._{50}$ and on the Slope of the l.d.r.l.

Times used previously	No. of tests	log. $C.D._{50}$ in mu./g. and S.E.	x^2 between tests	Slope of l.d.r.l. and S.E.	x^2 between tests
0	85	$\bar{1}.6842$ (0.012)	634.5	5.154 (0.148)	109.68
1	58	$\bar{1}.7316$ (0.012)	356.9	5.639 (0.181)	52.33
2	45	$\bar{1}.8023$ (0.010)	136.6	5.323 (0.206)	52.02
3	27	$\bar{1}.8038$ (0.014)	90.04	5.394 (0.266)	35.85
4	13	$\bar{1}.7867$ (0.025)	50.	4.782 (0.115)	4.73
5	3	$\bar{1}.7983$ (0.049)	11.38	4.549 (0.257)	2.53

	x^2 between usage 43.5				x^2 between usage 7.49	
Critical $x^2 P = 0.05$	$n = 84$	$n = 57$	$n = 44$	$n = 26$	$n = 12$	$n = 2$ $\quad n = 5$
	110.71	79.25	63.70	38.88	21.03	5.99 $\quad$ 11.07

A possible explanation of this is that the mice lost through deaths during the early tests are the more sensitive ones. It would also appear permissible to telescope the groups of mice after they have been used twice previously. The prior usage of mice has no significant effect on the slope of the l.d.r.l.

iii. *Selection on Basis of Sex.* For practical reasons the use of one sex is to be preferred.

The influence of sex on the reactions of mice to insulin during 231 tests has been examined. The summarized results are shown in Table XIX. They indicate that sex has some effect on the $C.D._{50}$ but not on the slope of the l.d.r.l.

TABLE XIX

Influence of Sex on the $C.D._{50}$ and on the Slope of the l.d.r.l.

Sex	No. of tests	log. $C.D._{50}$ in mu./g. and S.E.	x^2 between tests	Slope of the l.d.r.l. and S.E.	x^2 between tests
Male	182	$\bar{1}.7482$ (0.008)	1389.2	5.3093 (0.102)	198.88
Female	49	$\bar{1}.7106$ (0.013)	232.9	5.2863 (0.193)	65.75

	x^2 between sexes 6.3			x^2 between sexes 0.0108	
	Critical $x^2 P = 0.05$	$n = 181$	$n = 48$	$n = 1$	
		219.66	68.527	3.841	

E. PREPARATION OF SOLUTIONS FOR TEST AND THEIR INJECTION

It is convenient to administer all the doses of insulin in the same volume. The volume decided upon will depend on personal choice.

We feel it should not exceed 0.5 ml. and have preferred to make it 0.25 ml. per mouse.

It is customary to make the injections subcutaneously, but Trevan and Boock (1926) reported that they found intravenous and subcutaneous injections equally efficient.

F. TREATMENT OF MICE DURING THE TEST

After injection and for the duration of the test the animals are normally maintained at an elevated temperature. Trevan and Boock
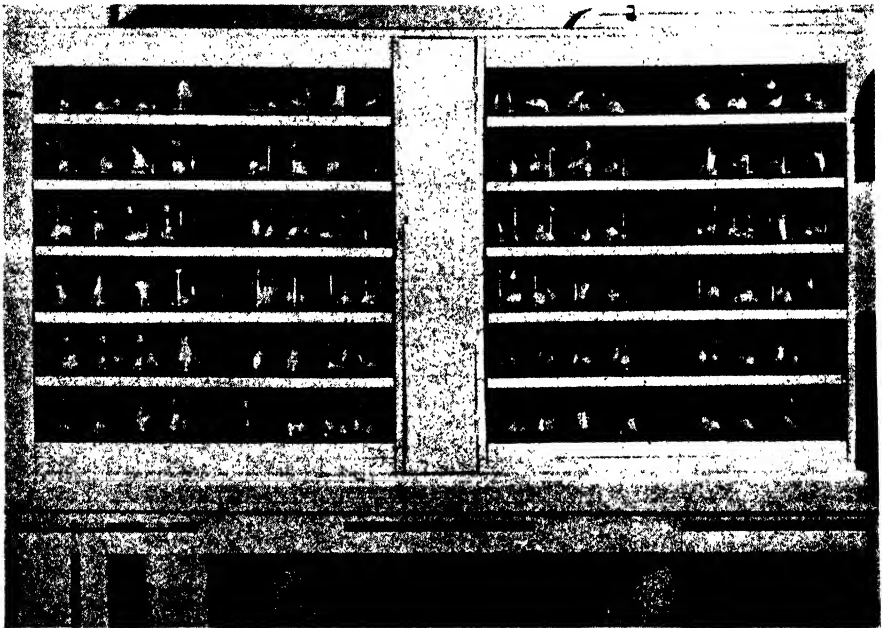
FIG. 4. An insulin testing cabinet for mice.

(1926) used for this purpose a thermostatically controlled water bath in which heavy containers were placed to hold the mice. Krogh and Hemmingsen (1926) described the use of an air incubator with a glass front, and it is with such an apparatus that we have been accustomed to work. It has the advantage that the mice may be observed during the test and those which convulse removed quickly and given a therapeutic injection of glucose.

Our testing cabinet (Fig. 4) is a shallow cupboard in which access to six shelves is provided separately by means of glass slides. It is warmed by electric heater wires fitted below each shelf, the temperature

is thermostatically controlled, and the air within the cabinet is mixed by means of a fan placed in the space at the rear of the shelves.

The cabinet holds 48 glass jars to house 96 treated mice; those jars which are to hold mice having the same dose are similarly colored and distributed throughout the cabinet so that the small temperature gradient which exists is applied equally to all doses. The position of these colored jars in the cabinet is constant and in each test the doses are assigned to the various colors at random.

Thorp (1948) has referred to the construction of a glass fronted cabinet of cubic form. Air warmed by passage over suitable heating pads is supplied through the roof of the cabinet by means of a fan, and the temperature is thermostatically controlled. The jars to hold the mice are placed on small movable racks, and the apparatus has the distinct advantage that it is readily cleaned.

Thompson (1946) has recently described the use of an inclined wire for the observation of mice showing the hypoglycemic reactions. This mesh screen 24 in. by 36 in. which will hold 100 or more mice is fitted at an angle of 60° and so arranged that the injected mice placed on it can only leave the screen over an edge which is 8 in. or more from any other surface. Mice with advanced symptoms fall from the screen into a tray from which they may be removed to be injected with glucose.

A modification has been described by Young and Lewis (1947) in which the screen is replaced by a wire mesh drum of 8-in. diameter and which is caused to revolve continuously, rotating once every 40 sec. Under these conditions the mice apparently lose their hold before severe hypoglycemia has developed and feed on a suitable diet in the receiving tray, so rendering the injection of glucose unnecessary.

G. TEMPERATURE FOR THE CONDUCT OF THE TEST

Elevated temperatures are necessary if mice are to exhibit the characteristic convulsions associated with hypoglycemia and use of varying temperatures for the assay of insulin in mice have been described. Trevan has reported results using a temperature of 37°C., Hemmingsen preferred a temperature of 29°C., for at this temperature he found that his losses of animals were less. For many years we have used a temperature of 32°C. at which temperature the mice did not display the signs of the discomfort shown at 37°C. and appeared to respond more consistently than when a temperature of 29°C. was applied. Differences in the slopes of the l.d.r.l. obtained in the various laboratories may have been attributed to the temperature used, but it is certain that other factors have also contributed to these differences.

Data supplied by Trevan and Boock (1926) working at 37°C. indi-

cated a slope of the order 5, but a review (Irwin, 1943) of data accumulated later in the same laboratory while this temperature was still maintained indicated a slope of 3.0. Hemmingsen (1933), working at 29°C., has recorded a slope of 4.9. In our own laboratory the mean slopes recorded for 269 tests during a period of 1944–1945 was 5.43 and in a more recent period 1948, the mean slope in 231 tests was 5.32. These tests were carried out at 32°C., the slope values are higher than those reported by Rowlinson and Lesford (1948) working at 34°C.

The use of wire meshes, either fixed or revolving, since it determines the onset of less severe hypoglycemic symptoms has particular application in the conduct of insulin assays at temperatures lower than those normally applied.

Even though temperature may not have marked effect on the slope of the l.d.r.l., and therefore on the accuracy of the assay, it is certain that it has marked effect on the level of sensitivity, and for this reason it is important that whatever temperature is applied should be controlled as closely as possible.

H. CONVULSIVE SYMPTOMS

The reacting mice mostly convulse violently; often, however, a mouse may pass into a state of collapse determined by the failure of the animal to right itself when placed on its back.

Both symptoms are considered equally positive. The animals are removed from the cabinet and injected with 0.5 ml. of a 15% glucose solution. They are not separated on removal, for the final score may be easily made by counting the empty spaces in the cabinet.

I. DURATION OF THE TEST

At 32°C. convulsions may commence within 20 min. of injection and reach their peak incidence in $\frac{3}{4}$ to 1 hr. but rarely occur after $1\frac{1}{2}$ hr. For this reason we have chosen to observe the mice for $1\frac{1}{2}$ hr. after injection. When the injection of one dose is completed, the time is noted and readings taken from that point. Generally speaking it is found that 15 min. should be allowed for the injection of 96 mice so that the overall time taken for a test is $1\frac{3}{4}$ hr.

J. FREQUENCY OF USE OF MICE

Animals on test on any day are fed at the completion of the test. It has been found that after a further 2 days of normal feeding they are suitable for test on the next day. Thus mice which have been used for test on Monday can be used again on Thursday.

K. NUMBER OF TIMES TO BE USED

It is our practice to use mice up to 6 times. Use on more occasions than this is seldom possible since at that time it is difficult to obtain sufficient mice of the correct weight range and "times used" to give full groups. The information in Table XVIII, however, suggests that segregation on the basis of usage may be abandoned after the mice have been used twice previously.

Table XVIII also indicates that during these tests 85 groups of new mice were taken and 231 tests conducted. This means under these conditions each mouse was used 2.7 times on an average, although the value would in fact be higher than this since some of the mice would have been used for other, approximate assays. The magnitude of this value will naturally depend on the amount of testing to be done since a larger float of animals and pressure of testing allows them to be used more efficiently. Estimates made on two other occasions indicated that on the average the mice are used 3.7 and 4.1 times. These two estimates were from periods in which the mice used were of one sex.

V. COMPARISON OF RABBIT AND MOUSE METHODS OF ASSAY

1. Agreement between the Methods

The results in four out of five laboratories obtained during the standardization of the second international standard against the first, suggested that the mouse method yielded results different from those obtained by the rabbit method. This has been explained by the fact that in this case comparison was being made between preparations of widely differing purity, and it has been considered that such differences should not occur when samples of similar purity were being examined.

A comparison of results obtained in one laboratory under these conditions using both rabbit and mouse methods of assay are shown in Table XX. The rabbit tests were twin cross-over tests and each was weighted inversely as the variance of the estimated potency, and this weight used to calculate a weighted mean potency. The individual mouse tests were weighted similarly.

For each sample the agreement of the individual results obtained by each method was checked by means of the χ^2 test and a further χ^2 test made to check the agreement between the two methods.

Only with sample H488 is there a somewhat wide difference between the mean potencies estimated by the two methods, but this is not significant at $P = 0.95$ level.

TABLE XX

Agreement between Rabbit and Mouse Methods of Assay

		Rabbit method				Mouse method					
Sample	Esti-mated potency	No. of rabbit re-sponses	No. of tests	Sw	χ^2 be-tween tests	Esti-mated potency	No. of mice	No. of tests	Sw	χ^2 be-tween tests	χ^2 be-tween meth-ods
H487	63.96	96	3	536.6	1.26	66.22	524	5	860.8	2.89	0.07
H488	168.5	160	5	2204.3	1.03	147.2	452	4	1259.0	1.88	2.76
H489	217.0	128	4	624.8	0.82	203.2	560	4	1182.1	1.49	0.33
H490	190.8	160	5	1845.1	8.41	195.4	1120	7	2199.7	8.08	0.11
H491	220.1	160	5	688.9	1.69	248.5	384	2	870.8	0.10	1.09
Sum		704		5899.7			3040		6372.4		

2. Relative Efficiency of the Two Methods

Ignoring all other factors which must be considered in assessing the relative efficiency of the two methods, the data summarized in Table XX indicate that the average contribution to Sw made by one rabbit = 5899.7/704 = 8.38 and that the corresponding contribution made by one mouse = 6372.4/3040 = 2.10. It would therefore be concluded in this instance that 4 mice had an efficiency equivalent to 1 rabbit.

From the point of view of the labor and space required for the housing and the feeding of the animals and for the conduct of the assay, reliance on the mouse method has distinct advantages, but perhaps its greatest value lies in the speed with which a result may be obtained.

Since, however, it is most probable that a colony of rabbits will be maintained in any case, the possibility of using this to the full to supplement the mouse method of assay must be considered.

This possibility will be approached with hesitancy if the method proposed involves blood sugar readings over 5 hr. and the use of a 7-day interval between the separate stages of the test. It becomes more attractive, however, when it is considered that each member of the colony could supply a response to insulin on each day if the response were measured by means of a single blood sugar reading taken 1–1½ hr. after injection.

VI. THE ASSAY OF INSULIN IN BLOOD

Although the use of the glucose-fed adrenodemedullated diabetic hypophysectomized (ADH) rat in assays of blood insulin has not yet been placed on a firm statistical basis, the results of Anderson *et al.* (1947) and Anderson and Long (1947a, b; 1948) are so promising that mention must be made of the technic.

The ADH rat is given 5 ml. of 20% glucose by stomach tube and then a subcutaneous injection of nembutal. It is then placed in a box at 101°F. for 25 min., and when well anesthetized is put on an operating board and the jugular vein is exposed. At the 29th min. the sample under test is injected into the vein and blood samples are taken from the tail 1 min., 15 min., and 30 min. later, i.e., 30, 45, and 60 min. after the glucose was given. The blood samples are assayed chemically for sugar, which falls in the presence of insulin. A fall of about 50% is produced by the injection of 0.001 unit of insulin, and detectable falls by as little as 0.000125 unit. An apparently linear log dose-response line is produced between these limits and would seem readily adaptable for accurate assay.

The technic has been used by Anderson and Long (1948) for detecting the presence of insulin in profusates from the isolated rat pancreas.

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Parathyroid Hormone

By R. H. THORP

CONTENTS

I. INTRODUCTION

The hormone from the parathyroid gland has not been isolated in a pure form, although extracts containing the hormone were first prepared in 1924. Little is known of the chemistry of this hormone although it is almost certainly a protein, since extracts give the characteristic protein reactions and the activity is destroyed after digestion with proteolytic enzymes.

The parathyroid hormone plays a major part in calcium and phosphorus metabolism, and in deficiency states an abnormally low concentration of calcium is present in the blood serum together with elevation of the renal threshold to phosphate excretion, there is also reduction in the amount of phosphate lost from the body in the urine. The most predominant effect of parathyroid hormone deficiency is an increase in the ratio of phosphate to calcium in the serum and, conversely, the administration of extracts of the hormone causes an increase in serum calcium together with a slight reduction in the phosphate ion concentration. Many methods used for the assay of parathyroid hormone

have been based upon the rise in blood calcium, and various devices
using this criterion have been employed, ranging from direct measurement
of the blood calcium concentration to the antagonism of magnesium anes-
thesia, which had previously been observed after injection of ionizable
calcium preparations.

The excretion of calcium in the rat has also been used as a basis for
an assay and Gellhorn (1935) has suggested the possibility of using the
sensitivity to calcium of hypodynamic skeletal muscle in an isolated
preparation as an indicator of parathyroid extract potency.

II. Possibilities of a Standard Preparation

There is no international standard for the parathyroid hormone,
and the assays which have been described either define the potency of
the preparation in terms of a response or by comparison with a private
standard, adopted by that particular laboratory, which has itself been
standardized in terms of a biological response.

It is now universally appreciated that a standard preparation of
similar constitution to that of the material under examination is essential
for any biological assay and the work of Dyer (1936) or L'Heureux et al.
(1947) may lead to the production of suitable material for this purpose.

Dyer described a stable powder which he suggested could be used
as a standard preparation and which he prepared in the following manner.
Fresh ox parathyroid glands were frozen and minced and then mixed with
picric acid. The mush was then covered with 10% acetone and left in a
refrigerator for 24 hr. After filtration the residue was pressed and
shaken with 70% acetone at 0°C. for a further period of 24 hr. and then
filtered again. The filtrates were bulked and the acetone recovered
in vacuo. The residue was dissolved in acid alcohol at pH 4.6–5.0,
the alcohol distilled off and the residue air dried and purified by solution
in hot phenol and reprecipitation from the cold solution with ether. The
solid material was washed with ether and stored at a low temperature
over phosphorus pentoxide. Dyer prepared four such batches and found
an activity of 80 Collip units/g. in each case.

L'Heureux and his colleagues following the work of Ross and Wood
(1942), who prepared extracts 2 to 3 times as potent as those of Collip
and Clark (1925), described a method for preparing a powder which is
soluble in water below pH 4.5 and has a potency of 200–300 U.S.P.
units/mg. of nitrogen. This material is similar in activity to that
obtained by Ross and Wood but is obtained in greater yields, a total
of 10,000 U.S.P. units, having been derived from 100 g. of fresh ox para-
thyroid gland. Electrophoresis examination of L'Heureux's material
showed it to be heterogeneous and to consist of two main components

but the separation of these and measurements of their relative activity has not yet been described.

For their assays L'Heureux *et al.* used a method (described later in this chapter) in which comparison was made with a commercial sample of parathyroid extract retained as a standard throughout their work, thus ensuring that the results they obtained were internally comparable.

III. The Unit of Parathyroid Activity

In the absence of a standard preparation the activity of parathyroid preparations has been stated in terms of the biological response and two units were described in this way.

Collip originally defined that unit as $\frac{1}{100}$ part of the amount of an extract required to raise the serum calcium of a dog weighing 20 kg. by 5 mg./100 ml., but Hanson (1928) proposed a smaller unit consisting of $\frac{1}{100}$ of the amount causing a rise of 1 mg./100 ml. in the serum calcium of parathyroidectomized dogs.

The unit described in the United States Pharmacopoeia (XIII revision) is similar to that of Hanson except that the rise in the serum calcium is that produced in normal dogs within 16–18 hr. after administration of the hormone preparation.

IV. Methods of Assay

1. Methods Based on the Elevation of Serum Calcium

A. METHODS USING DOGS

The method originally used by Collip and Clark (1925) involved the measurement of the rise in serum calcium after dosage with parathyroid extracts in groups of 10 dogs.

Blood samples were taken from the ear veins of 10 normal dogs of approximately 20 kg. weight, and the calcium content of the serum was determined. In the late afternoon of the same day (6.00 P.M.) the dogs were given a subcutaneous injection of the parathyroid extract under examination and the serum calcium was again determined at 9.00 A.M. the following day. The calculation of potency in this case was a simple arithmetic proportion based on a rise of 5 mg./100 ml. being produced by 100 u. of parathyroid activity.

The method originally described by Collip and Clark has been the subject of many modifications and improvements of which probably the most significant are the investigations of this method by Miller (1938) and Bliss and Rose (1940).

Miller first examined the dose-response relationship for the para-

thyroid elevation of serum calcium and showed, as one would expect, that the linear part of this relationship was small and corresponded only to a threefold change in dose. He found that when no account was taken of this relationship very variable results were obtained and one of the samples examined gave results varying from 75–222 U.S.P. units/ml. when the dose given was varied from 2–6 ml./dog. Efforts to extend the dose range, over which a steep response relationship would be obtained by modifications of the diet of the dogs, were without success. Miller concluded that the dose given must be one producing a significant response, which is submaximal, using a group of at least 5 dogs. In his experiments a rise of serum calcium of approximately 5 mg./100 ml. was a maximal response.

The examination of the method by Bliss and Rose (1940) followed upon the work of Miller and was directed principally toward determining the accuracy and reproducibility of the results obtained in such assays. These authors pointed out the great variation in sensitivity from dog to dog and the necessity for the use of a standard preparation. They examined data supplied by Miller as well as those derived from their own experiments.

Two experimental designs were used in the experiments which Bliss and Rose made, either 5 groups of 4 dogs each being tested 4 times in a Latin square arrangement, or 3 groups of 12 dogs each being tested twice in an arrangement of symmetrical pairs. In order to achieve comparable results the standard employed was that used in the Lilly Research Laboratories. Their investigations can best be discussed under several headings.

i. *The Dose-Response Curve for Parathyroid Extract.* Using Miller's data obtained on 12 dogs each treated with 7 doses of the extract ranging from 0.5 to 6.0 ml., and with determinations of serum calcium 7 hr. before and 17 hr. after injection, separate dose-response curves were plotted for each of 10 dogs which had contributed results all through the experiments.

The data used were both the absolute value of serum calcium after the hormone and the rise in serum calcium above the initial value for each dog. Using the latter data, Bliss and Rose showed that the slope of the dose-response curves varied from $b = 4.30$ to 9.49 with a combined value of 6.846. They calculated an analysis of the variance of the results and they showed that computations from responses within dogs were more consistent than between different dogs. The slope of the dose-response curve for individual dogs agreed within normal experimental limits.

When these results were plotted as the rise in serum calcium against

log dose and constant terms were added to reduce individual curves to a common basis, it was shown that the relationship was linear and maximal, for the extract employed, with doses of 6 ml./dog. The relationship obtained in this way could not be reconciled with the implied relationship of the U.S.P. (XI revision).

When only the final serum calcium data were used the combined slope was $b_c = 7.330$ and ranged from $b = 4.27$ to 10.22. In this case the variation between animals was more marked but the authors concluded that no criterion involving the initial serum calcium gave better results than using the final serum calcium value alone.

These findings were confirmed by the experiments conducted by Bliss and Rose themselves, and again, the final serum calcium level was a satisfactory criterion. Separate estimates of slope did not vary significantly but all the values obtained in these experiments were significantly less than those from Miller's data. The combined slopes in Bliss's experiment being $b_c = 4.133$ for the increase in serum calcium and $b_c = 4.082$ for final serum calcium alone, thus emphasizing the importance of an integral determination of slope in such an assay.

ii. *Suggested Assay Design.* Bliss and Rose proposed that a satisfactory assay should be a comparative test with a standard preparation, should have an internal determination of slope and error, and the difference in overall sensitivity should be separated from the estimates of potency and error. They proposed the use of a Latin square design to encompass these requirements (Bliss and Marks 1939a, 1939b.) and consequently investigated an arrangement of five 4x4 squares in parallel, with all 5 squares conducted on each day.

Analysis of variance showed that the difference between days was small, between dogs large, and once again, the final serum calcium value was an adequate criterion. The sums of squares for treatments were subdivided by factorial analysis, and it was shown that differences in dosage contributed mostly to the value, while the divergence from parallelism of the curves, for the standard and test materials, was of no significance.

With 40 observations on both standard and test preparations the standard error exceeded $\pm 10\%$ partly because of the smaller slope in Bliss and Rose's experiments and partly because the strength of the sample used as the test material in these experiments was 30% more potent than postulated.

Using the design of symmetrical pairs described by Yates (1936) these workers obtained a result with a standard error of $\pm 13\%$ (when the determined potency was 113% of that of the standard material) using 36 dogs with two tests on each. They described the application of this

design quite fully and also pointed out that unless the body weight of the animals is uniform throughout the group, it is essential to make a correction for the different values.

Using Miller's data, accuracy of a similar order was obtained with 11 dogs for both standard and test preparations, due to the steeper slope obtained in his laboratory.

The actual technic of the dog serum calcium method has been examined by Allardyce (1931) who stated that a meat diet with cod liver oil gave better results but that the addition of calcium as calcium lactate did not result in an increased response to parathyroid hormone. Since the more recent statistical designs were not current at this time, further work on these lines might reduce slope variation between dogs and reduce the number of observations required for a given degree of accuracy in this assay. Allardyce found incidentally that cats did not show appreciable hypercalcaemia after parathyroid injection and hence could not be used as test objects for this purpose.

The method for the determination of serum calcium requires little comment and a suitable one is described in the United States Pharmacopoeia (XIII revision).

B. THE USE OF RABBITS

Hamilton and Schwartz (1932) described a method using rabbits in which parathyroid hormone caused a delay in the rise of serum calcium after administration of calcium chloride by mouth.

Full-grown rabbits were used and the serum calcium was determined before and 15 min. after doses of calcium chloride (100 mg. Ca) given by stomach tube at intervals of 0, 1, 3, and 5 hr. Doses of 0.3–4.0 U.S.P. units of parathyroid hormone delayed the peak serum calcium rise until 3 hr. in the case of 1.0 unit, whereas in normal rabbits the maximum serum calcium value was observed after 1 hr. from the administration of calcium chloride and showed a gradual fall although subsequent doses of calcium were given.

These authors used the method merely as an approximate test for parathyroid hormone in biological substances and used as their criterion a serum calcium value greater than 4.00 mM./l. to indicate that the injected sample contained more than 1.5 units/dose.

Hamilton and Highman (1936) used this test to detect abnormally large amounts of parathyroid hormone in blood. Bauman and Sprinson (1940) also used this method but found that there was a marked species difference and that Dutch and Belgian rabbits were more sensitive than New Zealand white or Chinchilla rabbits which required approximately

three times as great a dose to produce the effect defined as positive by Hamilton and Schwartz.

Dyer (1935a) examined the method of Hamilton and Schwartz in a detailed manner and found that the variations in normal serum calcium values in rabbits and the values after parathyroid injections were very great so that it was not possible to give an average figure for the response to a given dose of parathyroid hormone or to compare the potency of two extracts by measuring the increase in serum calcium.

In two experiments in which 12 rabbits were used, graded doses of the same parathyroid extract did not produce a graded rise in serum calcium, and Dyer concludes that the method of Hamilton and Schwartz is of value as a means of detection of parathyroid hormone, but is not satisfactorily quantitative for assay purposes.

The methods based on elevation of serum calcium are therefore satisfactory when used as described by Bliss and Rose (1940) but can have little meaning without the use of a standard preparation and an adequate statistical design.

2. The Antagonism of Magnesium Anesthesia by the Rise in Serum Calcium Produced by Parathyroid Hormone

The reversal of magnesium depression by calcium ions has been known since 1905 (Meltzer and Auer 1905, 1908, 1913) and Simon (1935) showed that in normal mice subcutaneous injections of parathyroid hormone had the same effect upon magnesium narcosis as injections of calcium chloride due to the rise in serum calcium produced by the hormone. He also found that there was an optimal dose of parathyroid hormone which prevented magnesium narcosis in the greatest number of mice. Simon suggested the assay of parathyroid hormone by comparison of this optimal dose for different preparations in comparison with that obtained with a standard preparation.

Dyer (1935b) examined this method in detail, making use of the observations of Wokes (1931) who constructed a curve relating the percentages of mice affected by different doses of magnesium sulfate. When magnesium sulfate is injected subcutaneously into mice they become drowsy and unconscious, and unless a fatal dose has been given, respiration continues in a regular manner. Dyer used as his criterion of narcosis the inability of a mouse to right itself when turned on its back.

Dyer first investigated the effect of single and repeated doses of parathyroid hormone prior to magnesium narcosis and showed that a given dose of parathyroid extract was much more effective in antagonizing magnesium narcosis when injected in three increments at intervals of

3 hr., the dose totaling 0.09 ml. of parathyroid extract, than when given as a single dose. In fact a single dose had practically no effect on magnesium narcosis 2½ hr. later, in both cases over 80% of the mice remaining narcotized an hour later. The same dose given in three injections reduced the percentages narcotized at this time to below 10%. In this experiment also, Dyer showed that the effect of a total dose of 0.09 ml. of parathyroid extract given in two or three doses was greater than that produced by 0.18 ml. given as a single dose, although this produced a
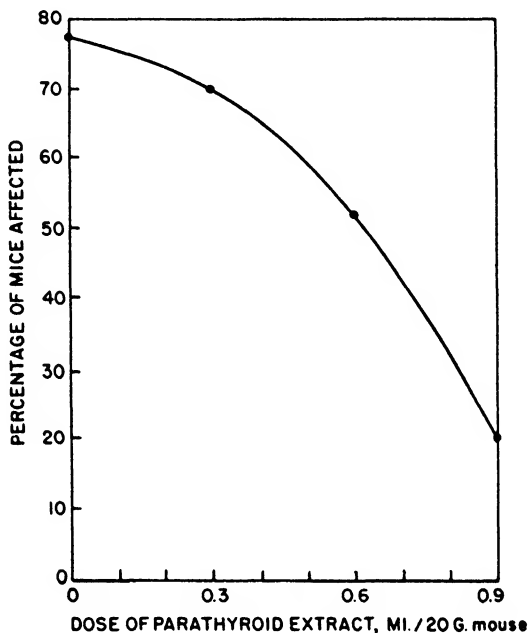
FIG. 1.   The effect of parathyroid extract in preventing narcosis in mice produced by injections of magnesium sulfate.   (Dyer, 1935b.)

more marked antagonism than a single dose of 0.09 ml. By using doses ranging from 0.3 to 0.9 ml. of an extract given in three divided doses to groups of mice at 2-hr. intervals, Dyer showed that the percentage of mice narcotized was related to the dose of parathyroid extract and a smooth curve could be drawn relating the two. Dyer's curve is reproduced in Fig. 1. For this experiment he used the percentage of mice narcotized 1 hr. after the injection of magnesium sulfate when this was given in a dose of 1.5 mg./g. of body weight, 1½ hr. after the last injection of parathyroid hormone.

Dyer compared the potency of two extracts, assayed by another method, by the use of this technic and although it happened that the

percentage of mice narcotized was the same in each case, and thus the potency was in inverse ratio of the doses given, the results agreed very well with that previously obtained. A statistical evaluation of this method has not been described, but it has the advantage of simplicity and rapidity and would appear worthy of extension.

3. Assay Methods Using the Fall of Serum Phosphate

Tepperman et al. (1947) were interested in assaying large numbers of samples of parathyroid hormone and fractions derived from it and found the serum calcium method in dogs too costly and time consuming for this purpose. These workers developed a method using the fall in serum inorganic phosphorus in the rat after parathyroid hormone and obtained a linear relationship between response and log dose over the range 12.5–100 U.S.P. units and a similar degree of accuracy to that obtained by the dog serum calcium method.

Tepperman and her colleagues describe two designs for the assay of parathyroid hormone by this method, one in which a previously established standard dose-response curve is used and a more complete design in which an integral determination of the slope of the dose-response curve is made by using two doses of each preparation.

The general technique, applicable in each case, consists of using groups of male albino rats fed on Purina dog chow for at least a fortnight before use, but not fasted prior to the experiment, although during the experiment only water is allowed. Blood samples are taken from the cut tip of the tail and 0.6 ml. is collected from each rat into centrifuge tubes, centrifuged for 10 min. and 0.2-ml. samples of the serum are pipetted into 6 ml. of 10% trichloro acetic acid, centrifuged and 5-ml. aliquots from the supernatant protein-free solution are used for the estimation of inorganic phosphorus by the method of Fiske and Subbarow. Tepperman et al. used an Evelyn photo electric colorimeter for the final reading of the color.

In their experiments these authors have used parathyroid extract (Lilly) as a standard in the absence of any official preparation and they measured the serum phosphorus initially and 3 hr. after subcutaneous injection of parathyroid hormone.

Figure 2 shows the dose-response curve obtained by this method and it will be seen that the relationship between log dose and response is substantially linear. The number of observations at each point varied from 121 at a dose of 50 U.S.P. units/rat to 22 in the case of the 100 units dose.

For assay of relative potency by the shorter technique these workers describe a design in which two doses of the test preparation are used

with group of 3 rats for each dose. The fall in serum phosphorus is
determined 3 hr. after injection and an adjustment made for differences
in the initial serum phosphorus level since it had been shown earlier that
this factor influenced the fall to a considerable extent and that regressions
of the fall in serum phosphorus on initial level had similar slope values for
different dose levels of the hormone. This adjustment corrects all
responses for an initial value of 9.15 mg. %, the mean value obtained by
these authors. The log ratio of the potencies of the unknown and
standard preparation is then calculated by the standard procedure using
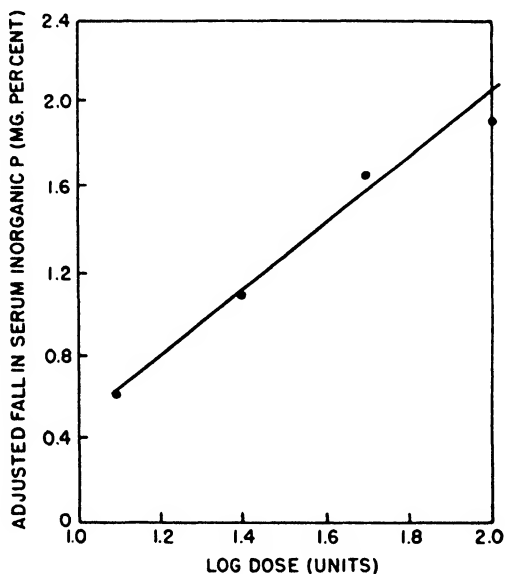
FIG. 2.   Dose-response curve obtained on rats using parathyroid hormone to produce
a fall in serum inorganic phosphorus.   (Tepperman *et al.* 1947.)

a modification which incorporates the use of a predetermined standard
curve. This modification involves a test of parallelism of the regression
lines for each preparation and correction for any difference which may be
found before calculating the variances of the mean response to the test
sample. The potency of the unknown sample and its standard error
can then be calculated. A sample assayed in this way gave a value
of 173 U.S.P. units/mg. of nitrogen with a standard error of ±33 U.S.P.
units.

In view of the crudity of many of the preparations these workers
were testing, such a method was adequate, but they also describe in
detail the complete assay mentioned earlier in which Yates's design
of "symmetrical pairs" is used. This design enables the rat serum

phosphorus method to give results comparable with the results obtained by Bliss and Rose (1940) by the dog method, but with much less technical effort and expense. In an experiment using 12 rats in the symmetrical pairs design calculation of confidence limits ($P = 0.95$) indicated that upon repetition, only 1 assay in 20 would be expected to give an estimate outside the limits 60–142%, the potency being 93% of that of the standard in this case. In Bliss and Rose's experiments 36 dogs gave confidence limits of 87–140% at $P = 0.95$ when the standard and unknown were of identical potency. Using the same number of rats, therefore, should produce results of accuracy comparable to those of the dog method.

The methods were found to be successful only with fed rats, the fall in serum inorganic phosphate being erratic and ill defined in fasted animals. This has not been further reported upon but may mean that increased sensitivity could be produced by dietary modifications.

4. Methods Based on the Excretion of Calcium in the Urine

In some preliminary experiments with parathyroid hormone Dyer (1932) found an increase in calcium excretion in the urine of the rat and tentatively suggested that this might form the basis for a method of assay. Later, however, Dyer performed a number of experiments in which no rise in calcium excretion was observed, and this observation was also made by Pugsley (1932) who found that daily injections of the hormone were necessary to ensure a rise in urinary calcium.

In a later paper Dyer (1933) described further experiments in which he used rats weighing from 120–160 g. in groups of 10 animals and fed upon a diet containing 1% calcium carbonate. The effect of parathyroid extract was much greater with the high calcium diet. The rats were housed in groups of 5 in metabolism cages with a mesh floor and a device to separate the urine from the feces. After an initial period of 4 days, during which daily estimations were made of the calcium excreted in the urine, injections of parathyroid extract were made once daily for 3 successive days and the urinary calcium excretion was again determined each day and for the fourth day. The normal excretion for such a group of rats Dyer found to be approximately 1.3 mg./day, and this rose to 7.8 mg./day when the parathyroid hormone was injected.

The test as Dyer used it comprised two groups of at least 5 rats and he used one group for each of the materials under comparison. The doses used were 0.4 ml. of an extract equivalent to Parathormone (Lilly), and by retaining the rats for a further period of 4 days after the last injection it was possible to perform a cross-over test by giving the two samples to opposite groups for another period of 3 days.

The relationship between the dose of parathyroid hormone and the

rise in calcium excretion had not been fully established but available
evidence indicated it to be a linear one.

Truszkowski *et al.* (1939) reinvestigated methods for the assay of
parathyroid hormone and considered that Dyer's method could be useful.
They used larger rats weighing 150–200 g. and housed them in groups
of 5 in glass metabolism cages. The diet used in this case contained
approximately 0.3% of calcium carbonate and was fed to the rats in
different cages once daily.

These workers found the normal calcium excretion to be 0.404 mg./
100 g./day and they showed that the standard deviation of the difference
between two groups treated at the same time was less than half that for
the same animals at different times so that they suggested that the rise
in the difference in calcium excretion between two similar groups of rats
at the same time is more significant than for the same group of rats at
different times.

The method favored by Truszkowski and his colleagues, therefore,
was to record the daily excretion of calcium for about 7 days and then
to give an injection of parathyroid hormone to one of two groups. This
produced a steep rise in calcium output followed by a rapid fall, and the
extra excretion was complete in 72 hr. The control group was then used
to provide a baseline for the treated group, and the area of the curve
due to the injection of parathyroid hormone was plotted against the dose
given.

It was shown that there was a linear dose-response relationship for a
6X change in dose, although the exact doses given cannot be stated since
the extract used was not described in terms of a unit but given by weight.

Divided doses were tried, but although the response obtained was
greater Truszkowski *et al.* did not consider the increase outweighed the
extra work involved.

5. Gellhorn's Work with Parathyroid Hormone on Hypodynamic Muscle

A survey of methods for the assay of parathyroid hormone could
hardly fail to include the interesting observations made by Gellhorn
(1935) which might be adapted for assay purposes.

Skeletal muscle is highly sensitive to calcium ions when in a hypo-
dynamic state, and Gellhorn set up hind-limb preparations of Rana
esculenta arranged for perfusion through the abdominal aorta, and in
some experiments each limb separately via the iliac arteries. The
tendons of the gastrocnemius muscles were attached to isotonic levers
magnifying the contraction 7 times. Nerve stimulation was by con-
densor discharges at a rate of 40/min. until the height of contraction was
reduced by 50%. After 15–30 min. rest, stimulation was resumed at

15/min. and the preparation was used for experiments as soon as it had settled to a steady response.

Gellhorn used dilutions of 1 in 100 to 1 in 1000 of Parathormone (Lilly. 20 Collip units/ml.) in phosphate-buffered Ringer's solution of pH 7.2 and found that there was always an increase in the height of contraction and this increase was graded according to the concentration of the hormone.

The effect took about 2–5 min. to commence, and periods of 5–10 min. stimulation were used alternately with the same periods of rest. Gellhorn did not find this effect when the hormone was inactivated and, although it had been shown earlier that similar effects could be produced by calcium ions this was not the mechanism in Gellhorn's experiments. He therefore concluded that the hormone aided recovery from fatigue and augmented the height of contraction by its action in raising the calcium level.

The method has not been developed for assay purposes but might well be worthy of further consideration as it would have the advantage of being a much quicker method than any of the others described in this chapter.

In conclusion it would seem that the methods used by Bliss and Rose on the dog, or the methods of Tepperman *et al.*, Truszkowski *et al.* are all suitable assay procedures but that the necessity for a stable standard preparation of parathyroid hormone is an essential before uniformity of products and ease of comparison can be assured.

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Biological and Chemical Assay of Adrenalin

BY G. B. WEST

CONTENTS

I. INTRODUCTION

Adrenalin, the active substance obtained from extracts of the suprarenal medulla, is a chemically defined substance, so that it may at first sight seem strange that a biological method of estimating it should be considered. It is, however, submitted to a biological test in at least two circumstances: (a) for routine work, when a sample of adrenalin is to be tested to see if it has the full activity of the pure substance; and (b) for research work, when small quantities which cannot be estimated chemically are to be detected and measured. The following list shows the methods in common use:—

(a) Routine testing
  (1) The vasopressor action in cats or dogs
  (2) The inhibitory action on an isolated rabbit intestine
  (3) Shaw's chemical method and specific test

(*b*) Research work
    (4) Straub frog heart
    (5) Perfused frog heart
    (6) Perfused frog blood vessels
    (7) Hen's rectal caecum or pigeon's rectum
    (8) Perfused rabbit's ear
    (9) Isolated rat's uterus
   (10) Fluorescent reaction
   (11) Other chemical methods

In all biological assays of adrenalin, a freshly prepared solution of the pure material in weak acid, e.g., $N/100$ HCl, is normally used as the standard preparation. Such a solution (1 in 1000 of base) is quite stable for at least one month, if it is kept in the cold, protected from light and stabilized with 0.1% of sodium metabisulfite (Berry and West, 1944; West, 1945, 1946, 1947d). Dilutions of even 1 in 100,000 with distilled water (not saline) are stable for some hours and can be used for addition to Ringer's fluid to give the desired final concentrations for experiments. Such additions should be made immediately before use.

II. The Blood Pressure of a Cat or Dog

1. Intact Preparation

Full-grown male cats or dogs about 3 kg. in weight are the most suitable. The animal is anesthetized with ether or chloroform, secured on the operating table, and the trachea is exposed. A tracheal cannula is inserted so that artificial respiration may be applied when required. The carotid artery on one side is cannulated, connected to a mercury manometer, and arranged to record the blood pressure of the animal upon a kymograph. The femoral vein is exposed and atropine sulfate (1 mg./kg.) injected to paralyze the vagal receptor mechanism. The animal is then left for half an hour. In place of continuous ether anesthesia, some workers prefer to inject chloralose intravenously (50 mg./kg.) and then remove the ether connection. Chloralose animals exhibit steady blood pressures over long periods of time (up to 24 hr.).

2. Spinal Cat Preparation

Elliott's method is usually employed. Having inserted the cannula in the trachea (as described above), both carotid arteries are then ligated. The cat is turned over on to its abdomen, the legs are secured, but the head is left free. The next procedure is to expose the spinal cord beneath the long spine of the second cervical vertebra. The skin is divided by a

scalpel down the midline from the top of the head to the shoulders. The two flaps of skin are held back on each side. The worker then holds the cat's head in one hand so as to extend the muscles of the back of the neck and divides the first layer of muscles down the midline from the skull for about 6 to 7 cm. These muscles are held back, and further muscles from the sides of the spine of the second cervical vertebra are cut away. The muscle is cleared from the bone by using a thin, blunt dissector. The muscle attached to the lower end of the spine is cut through, and the whole of the spine may be cut off with bone forceps. At this stage, it is important that the cat should be as deeply anesthetized as possible in order to minimize the loss of blood. With a small pair of bone forceps, the layer of bone covering the spinal cord is nibbled away until the dura mater is exposed over a length of about 1.5 cm. and over its full breadth. The brain is rapidly destroyed by a probe thrust through the foramen magnum. When the probe is withdrawn, a tapering cone of plasticine is thrust into the brain in its place. Finally the foramen magnum is plugged with a small cork. Anesthesia is discontinued and artificial respiration is begun. After removing stray portions of plasticine from the spinal canal, the upper end of the cord should be seen separated by almost 1 cm. from the lower end. Some bleeding may occur from the end of the cord, but this is soon arrested by applying cotton wool dipped in warm saline. The skin of the back of the neck is sewn together with string, and the cat is turned on to its back once again. One cannula is inserted into the left common carotid artery for recording the blood pressure, and another into the right external jugular or femoral vein for making injections. It is best to leave the cat for an hour or more to settle down.

3. The Test

The first step is to test the uniformity of response and the sensitivity of the animal and so determine the most suitable dose of standard solution with which to compare the unknown. In the intact cat, the aim is to produce a rise in blood pressure of 30 to 60 mm. Hg, with a difference in dosage of 20% of the standard solution producing a difference of blood pressure of at least 5 mm. Hg. Alternate doses of the standard and unknown preparations may be given every 5 min. The doses chosen in the spinal cat preparation must produce a submaximal effect, yet should not be far from the maximal dose, since in this region the response to a given dose is more constant than elsewhere. In each comparison, a dose of standard should be found which is greater than a dose of unknown, and a dose of standard should be found which is less than a dose of unknown, the blood pressure rises being measured to the mm. Hg.

From the mean of the results, the strength of the unknown solution can be calculated. Differences of 5–6% can be accurately measured (Fig. 1). The biological assay of adrenalin in the U.S.P. XII is carried out on the blood pressure of an anesthetized intact dog, and evidence of potency within 5% below and 5% above the standard is acceptable. Pretreatment of the intact animal with ergotamine tartrate (0.1 mg./kg.) makes the pressor response more distinct owing to exclusion of the mechanical presso-regulation mechanism.
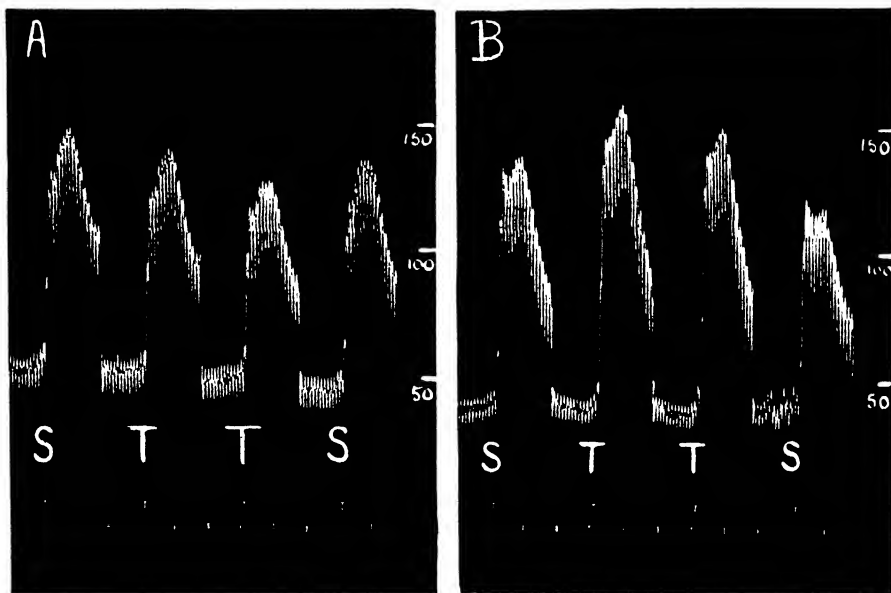
FIG. 1. Spinal cat 3.0 kg. Blood pressure record. (A) 1.0 ml. of unknown sample (T) less than, and (B) 1.1 ml. greater than, 10 μg. standard adrenalin (S). Time in 30 sec.

III. THE RABBIT'S INTESTINE

A strip of intestine from a freshly killed rabbit is suspended in an isolated organ bath containing oxygenated Tyrode's solution, maintained at 35–36°C. The rate at which oxygen is bubbled through the solution must be carefully controlled, for too rapid a stream interferes with the regular rhythm of the intestine and increases oxidation of the adrenalin. The length of the strip should be 3–5 cm. and the lever should be weighted so that the intestine always relaxes to the same point. If the amplitude of the contractions is too small, it can be increased by adding pilocarpine to the Tyrode's solution (0.1 mg./l.). Strips of the ileum are usually the most sensitive to the action of adrenalin, and

inhibition of intestinal movement will follow when the concentration of adrenalin in the bath is as small as 1 in 10 millions. Alternate doses of the standard and unknown preparations may be given every 2 min. (Fig. 2). This allows 1 min. for recording the action of the drug, and 1 min. for washing and recovery. Differences in dosage of 10% can be measured by this method.
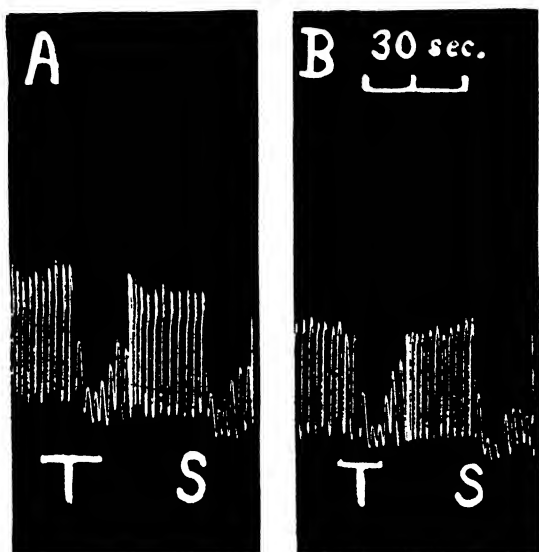
FIG. 2. Rabbit ileum. The unknown sample (T) produced relaxation just less than that shown by 1 μg. standard adrenalin (S).

IV. SHAW'S CHEMICAL METHOD AND SPECIFIC TEST

The method depends on the fact that adrenalin reduces arsenomolybdic acid with the formation of a blue color. It is a modification of Whitehorn's (1935) method but is simpler and more sensitive. Brief preliminary treatment of adrenalin with alkali in the presence of oxygen increases the color considerably (2 to 5 times), and this increase is specific for adrenalin or other phenols with the same side chain.

The solution to be tested (containing 0.1 to 0.5 μg. of adrenalin) should be neutral or faintly acid. It is placed in a centrifuge tube, 2 drops of phenolphthalein reagent are added and it is then carefully neutralized with 4% NaOH. One drop of N H_2SO_4 is added (approx. pH 4.0), then 2 ml. of the suspension of $Al(OH)_3$ (made fresh from potassium alum and sodium hydroxide). The solution is shaken and centrifuged for 2 min. The supernatant fluid is poured into another tube, and 1 ml. of $Al(OH)_3$ per 5 ml. of solution and 1 drop of phenol-

phthalein are added. Caustic soda is now added drop by drop with shaking until the solution is just distinctly pink (pH 8.5). The solution is shaken and centrifuged as before. The supernatant fluid is discarded, and about 3 ml. of water, made just alkaline to phenolphthalein with NaOH, are poured on to the precipitate; it is centrifuged and the fluid discarded, 2 ml. of water and 0.35 ml. of 4% NaOH are added to the Al(OH)$_3$, which should now go into solution.

The solution is kept for 2 min. and then 2 ml. of the H$_2$SO$_3$ reagent (fresh Na$_2$SO$_3$ solution and H$_2$SO$_4$) are added, and the mixture poured into a tube in a boiling-water bath which contains 0.7 ml. of the arseno-molybdic acid (sodium molybdate, sodium arsenate, and concentrated H$_2$SO$_4$) which has been heating for 5 min. After exactly 5 min., the tube is removed and placed in a beaker of cold water. The volume is made up to 5.5 ml. and the color is estimated in a colorimeter 15–20 min. later. The color does not fade but remains constant for at least an hour and then tends to increase slowly.

A blank is prepared by taking the same volume of Al(OH)$_3$ suspension as has been used in the test, adding 2 drops of phenolphthalein, making just alkaline with 4% NaOH and centrifuging. The supernatant fluid is discarded, 2 ml. of water, and 0.35 ml. of 4% NaOH are added to the Al(OH)$_3$, then the H$_2$SO$_3$, etc., as before.

A standard blue solution is prepared by subjecting 2 ml. of an appropriate solution of adrenalin to the first and second adsorptions with Al(OH)$_3$ and subsequent treatment with alkali, sulfite, and arseno-molybdic acid as described above. The color due to the blank must be subtracted both from that due to the unknown and that due to the standard. The curve showing the relation between the amount of adrenalin and the color is approximately linear for small, but not for large, amounts of adrenalin. But for greater accuracy, it is better to compare the color due to unknown solutions with that due to adrenalin solutions treated with the same reagents on the same day. The smallest absolute amount of adrenalin which can be estimated is about 0.04 µg., and the lowest concentration in which this amount can be estimated is 1 in 5×10^8. The standard deviation of a single observation is about 0.015 µg. Satisfactory agreement exists between the results of this test and that on the cat's blood pressure (Shaw, 1938).

The specific test requires the solution to be divided into two. One half is treated as above and the other in the same way except that the 0.35 ml. of 4% NaOH which is used to dissolve the Al(OH)$_3$ after the second adsorption is replaced by 0.35 ml. of water containing 1 drop of 1N H$_2$SO$_4$. The ratio of the color given by the alkali-treated half of the solution to that given by the untreated half is from 2 to 5.

V. THE STRAUB FROG HEART

In a pithed frog, a very fine cannula is placed in the right aorta and passed through the bulbus arteriosus into the ventricle. It is fixed in position by a thread round the two aortae. The heart is then removed from the animal and clamped in the upright position. Blood is washed out of the ventricle, and the heart is filled with Locke's solution diluted
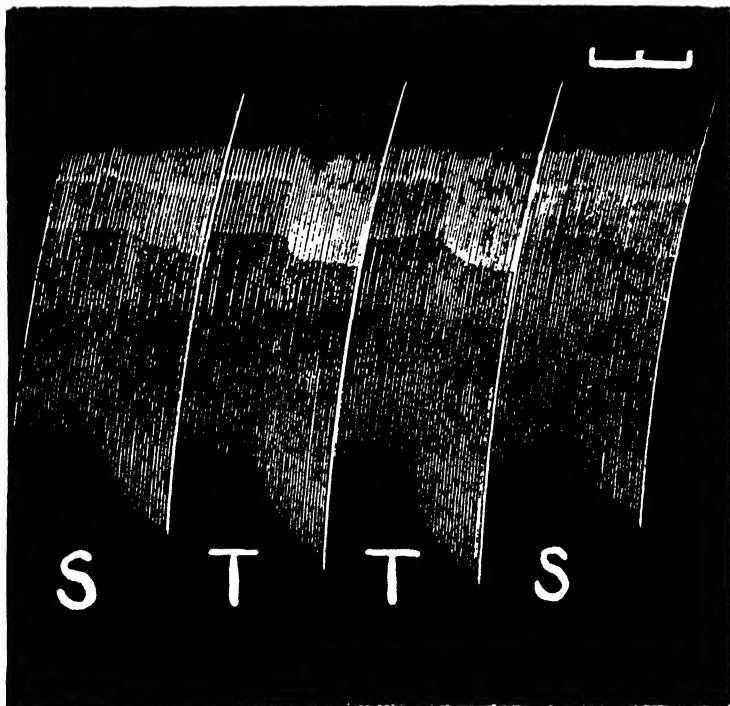
FIG. 3. Straub frog heart. Downstroke is contraction. The unknown sample (*T*) produced stimulation just greater than that shown by 10^{-9} standard adrenalin (*S*). Time in 30 sec.

to 1.4 times its volume with water, or with Clarke's Ringer solution. It is customary to include atropine sulfate (10^{-6} to 10^{-7}) in the liquid in the cannula, which is pumped up and down by each heart movement and is in contact with the heart muscle continuously. This movement is recorded on a kymograph by means of a thread and a long isotonic lever. The preparation usually reacts with an increase in the amplitude of the beat to a concentration of 10^{-9} of adrenalin and sometimes to a concentration of 10^{-10}. Nevertheless, it is not a good test object by

itself, since it is also sensitive to many other drugs and substances present in tissue extracts.   All dilutions of the standard and unknown solutions must be made in the same liquid as is used to bathe the heart.   The effect of one of these dilutions on the heart is observed by removing as much of the fluid from the cannula as is possible, replacing it by the solution under test, and allowing the drug to penetrate for a given period of time (usually 3 to 5 min.).   Then it is replaced by the normal perfusion fluid, and the amplitude of heart beat is allowed to return to its initial level.   It is then ready for the next dilution, and doses of the standard and unknown preparations producing equal stimulation are compared (Fig. 3).   The exterior surface of the heart is constantly moistened with Ringer's solution to prevent drying.   Differences in dosage of 10–15% can be detected by this method.

VI. The Perfused Frog Heart

1. Modified Symes's Method

Having exposed the heart of a pithed frog free from pericardium and having ligated the anterior venae cavae, a mammalian venous cannula, full of frog Ringer's solution, is inserted into the posterior vena cava.   The heart is then removed from the animal and the movements recorded on a kymograph by means of a thread and a long isotonic lever.   The cannula is connected to a perfusion apparatus in which the Ringer's solution in the reservoir is maintained at a constant level by a Mariotte bottle.   By use of another reservoir and a 3-way capillary tap (West, 1943), the perfusion of a dilution of the standard adrenalin solution in place of the Ringer's solution can be effected.   The perfusion is allowed to take place for exactly $1\frac{1}{2}$ or 2 min., after which the lever is removed from the drum.   The tap is turned to allow fresh Ringer's solution to perfuse.   A dilution of the unknown adrenalin solution is then prepared, during which time the heart beat has nearly always returned to normal. This dilution is perfused for the same fixed time, at the end of which it is replaced by Ringer's solution.   The unknown adrenalin is repeated, using the same dose, and finally the standard, so that a group of four results is obtained (Fig. 4).   It is found that the response to a given dose is more constant in the region of the maximum response than elsewhere. With most hearts, an adequate response is given by a concentration of 10^{-8} adrenalin.   Assays by this method on heated adrenalin solutions give results comparable with those obtained by the standard cat method (Berry et al., 1944).   The standard error of the test is 2.17%.   Limits of error ($P = 0.99$) are therefore 94 to 106%.
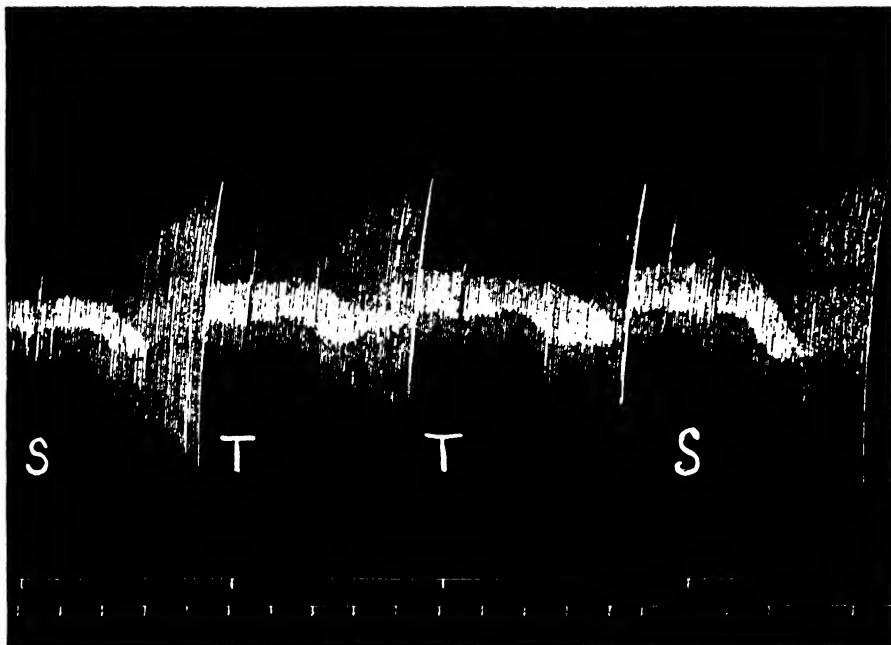
FIG. 4. Perfused frog heart. Downstroke is contraction. The unknown sample (*T*) produced stimulation less than that shown by 10^{-8} standard adrenalin (*S*). Each perfusion lasted for 2 min. only. Time in 30 sec.

2. An in situ Method

In this method, one cannula is tied in the vena cava of a pithed frog and another in the left aorta, the right aorta being tied. The heart is perfused at a constant pressure with frog Ringer's solution containing atropine 10^{-5} from a Mariotte bottle. The arterial outflow tube is fixed at a constant height of 4 cm. above the level of the heart. The apex of the heart is connected to a long light straw lever with a fine silk thread. The whole heart preparation is submerged in a dish with Ringers' solution just covering the up-stretched heart. The Mariotte bottle is kept at a fixed height so that the outflow of the heart is 1 drop per beat. The preparation is ready for use after 4 hr. perfusion (Bülbring, 1944). Alternate injections of dilutions of the standard and unknown adrenalin solutions are made close to the venous cannula by way of a rubber cap containing a small air bubble, which separates the fluid coming from the reservoir from the fluid in the mouth of the cannula (Gaddum and Kwiatkowski, 1938). Injections are made through a rubber cap. The injected fluid then cannot mix with fluid coming from the reservoir, and it is easy to control the rate of injections so as to avoid pressure changes

by watching the fluid in the mouth of the cannula.   An adequate stimulation of the heart beat is given by doses of 0.01 μg. of adrenalin.   Differences in dosage of 10–15% can be detected by this method.

VII. The Perfused Frog Blood Vessels

The perfusion of frog legs was developed by Laewen and Trendelenburg and has been modified subsequently in several details.   A satisfactory method consists of ligating the two anterior venae cavae and the right aorta of a pithed frog, and perfusing the blood vessels of the legs and
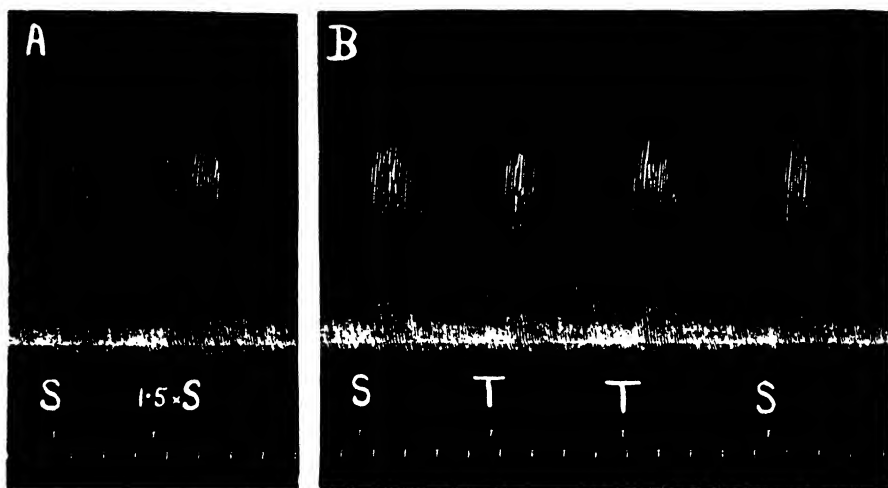
FIG. 5.   Perfused frog blood vessels.   Outflow record; the height is proportional to the time interval between drops.   A. Response graded according to the dosage. B. The unknown sample (T) produced vasoconstriction just less than that shown by 0.05 μg. standard adrenalin (S).   Time in 30 sec.

viscera with Clark's Ringer solution from a Mariotte bottle (West, 1947a). A cannula is placed in the left aorta, and the outflow is collected from a cannula in the posterior vena cava and measured on a drop timer.   After about 4 hr. perfusion, during which time the preparation becomes much more sensitive to adrenalin, consistent responses to 0.01–0.05 μg. are obtained.   Injections of dilutions of the standard and unknown adrenalin solutions are made close to the arterial cannula by way of a rubber cap containing a small air bubble (see under VI, 2).   The presence of edema is not detrimental to the responses, which are graded according to the dosage (Fig. 5).   The perfused blood vessels of female Winter frogs provide the most suitable test objects.   The vasoconstrictor effect will, however, only detect differences in dosage of 10–15%.

VIII. The Hen's Rectal Caecum

The fowl's rectal caecum is sometimes used to test whether an unknown substance acts like adrenalin in inhibiting this preparation, which is the most sensitive of the tissues known to be inhibited by adrenalin. The effective concentration is 10^{-9} and sometimes 10^{-10} (Barsoum and Gaddum, 1935). To economize material, the rectum may be suspended in a small bath of 2 ml. capacity, and kept at 30°C., since according to Blaschko and Schlossman (1938), this increases its sensitivity and diminishes the frequency of large spontaneous movements which are likely to spoil the record. Solutions of the unknown and standard preparations are warmed to this temperature before being added to the bath of Tyrode's solution containing half the normal concentration of KCl (Gaddum, Jang, and Kwiatkowski, 1939). Differences in dosage of 25–30% can be detected by this method. Similar test objects are those of the rectum of a week-old chick which is inhibited by 5×10^{-10} adrenalin (Mann and West, 1950), and of the pigeon's rectum which is inhibited by 10^{-9} adrenalin (Bülbring, 1944).

IX. The Perfused Rabbit's Ear

Large rabbits (over 3 kg.) with large ears are anesthetized with ether, and the common carotid artery is dissected out on one side. The external carotid artery is tied about 0.5 cm. above the carotid sinus, and all the branches central to this point are tied and divided, except the artery to the ear which runs laterally from the carotid at about the level of the superior cervical ganglion. This artery is left undisturbed in order to avoid damage to the postganglionic sympathetic nerves which run near it. The cervical sympathetic nerve is then traced up to the superior cervical ganglion and the postganglionic nerves which run laterally from the lower end of the ganglion are carefully dissected out for about 0.5 cm. The great auricular vein is then freed. After this preliminary dissection, during which time the blood supply to the ear is undisturbed, the arterial cannula is tied in the carotid artery and perfusion with Tyrode's solution from a reservoir is started (Gaddum and Kwiatkowski, 1938). A piece of thin glass tubing is tied in the great auricular vein as a venous cannula. A strong ligature is then tied round the neck, and the head is removed from the body. Blood and perfusion fluid are allowed to drain for some time from the region of the vertebrae. When the blood appears to be all washed away, the vertebral canal is blocked with plasticine, and the rate of outflow from the ear is measured on an outflow recorder. It is an advantage to equilibrate the Tyrode's solution with 5% CO_2 in O_2. The rate of outflow is kept constant by raising the perfusion reservoir to a height of 2–3 meters and interposing a capillary resistance between it

and the ear. Perfusion at a temperature of 37°C. does not seem to have any special advantage. Alternate injections of dilutions of the standard and unknown adrenalin solutions are made close to the arterial cannula by way of a rubber cap containing a small air bubble. The blood vessels of the rabbit's ear respond regularly with vasoconstriction to the injection of adrenalin 10^{-8} and over. Differences in dosage of 15–20% can be detected by this method.

X. THE ISOLATED RAT'S UTERUS

One horn of the non-pregnant uterus of an adult rat is suspended in Tyrode's solution containing only one-quarter the normal amount of calcium, i.e., 0.006% $CaCl_2$. The assay, best completed at 29–30°C. (de Jalon et al., 1945), is accomplished by adding to the bath constant amounts of acetylcholine (10^{-6}) every 2 or 3 min. and finding how these induced contractions are reduced by alternate doses of dilutions of the standard and unknown adrenalin solutions, given 1 min. before the next acetylcholine dose is due. The adrenalin effect is observed with as little as 10^{-9}. Differences in dosage of 15–20% can be detected by this method.

XI. THE FLUORESCENT REACTION

When adrenalin in simple alkaline solution is exposed to oxygen, a green fluorescence develops and then disappears. Attention was drawn to the advantages of this fluorescence for the detection of adrenalin by Gaddum and Schild (1934), who found that it provided a sensitive physical test by which a concentration of 10^{-8} adrenalin could be easily detected. They showed that oxidation is the cause of the fluorescence, since none was produced if all oxygen was removed from the solution. They recommend the addition of 0.1 ml. of 5N NaOH to 1 ml. of the standard or unknown solutions of adrenalin. The comparison of the intensities of the fluorescence is made by observing the solutions directly from above, when they are illuminated horizontally by light from a mercury vapor lamp, filtered through a glass which absorbed practically all visible rays. The reading is taken after 20 sec. contact, for after about 1 min. no fluorescence remains if adrenalin solution (10^{-8}) is used.

The difficulty implied by this method is the short time in which the intensity of the fluorescence is maximal and during which the reading has to take place. In an effort to place this test on a more quantitative basis, Jørgensen (1945) suggests the addition of 0.3 ml. of 2N NaOH to 2 ml. of the adrenalin solution, the intensity of fluorescence being read 1 min. after the addition of alkali. The comparison is made with a

similar tube containing 2.3 ml. of distilled water, to which small quantities of an eosin solution (0.1 mg. %) are added so that after 1 min., at the maximum of the reaction, the same intensity of fluorescence is seen in the two tubes exposed to ultraviolet light from a mercury vapor lamp. A standard curve can be plotted by titration, employing pure adrenalin solutions of known concentration, with the amount of eosin solution measured in 0.01 ml. plotted along the ordinate, and the adrenalin concentration in μg. % along the axis of abscissas. From the curve, the adrenalin concentration in the unknown solution corresponding to the amount of eosin used can be read. In practice, this method gives the best results when the adrenalin concentration is between 5 and 20 μg. %. The standard of error of the test is 2.15%, so that the limits of error ($P = 0.99$) are 94.5 to 105.5%.

West (1947a) recommends that the comparison be made in a simple fluorescence comparator, in conjunction with a Wood's glass to absorb practically all visible rays, with eosin solutions whose fluorescent intensities correspond to alkali-treated 10^{-7} and 10^{-8} adrenalin respectively. By using 3 ml. of the adrenalin solution and 0.3 ml. of $2N$ NaOH, calibration curves can be constructed to show that a linear relationship exists between fluorimeter readings and the concentration of adrenalin in the solution. Monax glass test tubes are used in this work, as they do not fluoresce in ultraviolet light. Estimates by these fluorimetric tests agree well with physiological and chemical values for pure adrenalin solutions and for the concentration of adrenalin in normal rabbit's blood (West, 1947c). The standard error of the test is 2.11%, so that the limits of error ($P = 0.99$) are 94.6 to 105.4%.

XII. OTHER CHEMICAL METHODS

A great number of colorimetric tests for adrenalin, varying widely in sensitivity and specificity, have been devised. The most widely used is that of Folin et al. (1913), which is simple and rapid. The phosphotungstic acid reagent gives a blue color with polyphenols and uric acid, and its use here is therefore dependent upon the presence of the two phenolic groupings in adrenalin. For this reason, it is frequently found that colorimetric estimates of decomposed adrenalin solutions are higher than those obtained by biological assay. However, the reagent provides a suitable method for the estimation of simple solutions of adrenalin. Folin and Ciocalteu's phenol reagent containing phosphomolybdic acid, which is sensitive to all phenols, is also useful for rapid and accurate determinations of adrenalin solutions of strength greater than 10^{-5}. Micro methods have been adopted for both assays (Somers and West, 1944). Briefly, the unknown adrenalin solution is

added to the reagent, saturated sodium carbonate solution being added to the mixture. The tube is immersed in a boiling-water bath for exactly 2 min., and then cooled. The color produced is compared in a colorimeter with the standard adrenalin solution similarly treated. The limits of error ($P = 0.99$) of these tests on pure solutions of adrenalin are 91.5 to 108.5%.

Most of the other chemical methods involve the formation of the unstable red oxidation product, adrenochrome, and references to these will be found in the paper by Barker et al. (1932). Colorimetric methods, however, due to their non-specificity, cannot entirely replace the physiological methods when the composition of the solution is unknown.

XIII. NORADRENALIN

It has been established during recent years that the pressor activity in extracts of the suprarenal medulla is not due to the presence of adrenalin only, but also to the presence of its primary amine, noradrenalin. This has been shown for the pig's gland by Schumann (1949) and for the dog's gland by Bülbring and Burn (1949a) by biological estimation. That both substances are present in extracts of adrenal glands from cattle was shown by Euler and Hamberg (1949) who compared the results obtained by biological methods with those obtained by paper chromatography (James, 1948). Noradrenalin has been demonstrated to occur in various organs and tissues of the body (Euler, 1948), and in medullary tumors (Holton, 1949; Goldenberg et al., 1949).

Bülbring and Burn (1949b) showed that in the eviscerated cat stimulation of the splanchnic nerve to the suprarenal gland caused release of a mixture of adrenalin and noradrenalin. The pressor effect of splanchnic stimulation could be matched by an infusion of adrenaline, but the ratio of contractions of the two nictitating membranes, one denervated and one normal, could only be matched by infusing a mixture of adrenalin and noradrenalin. This result was confirmed by West (1950) by injecting the blood from the suprarenal vein of a cat under chloralose (following stimulation of its splanchnic nerve) directly into the arteries supplying the denervated nictitating membrane and the denervated non-pregnant uterus of another cat under chloralose and cocaine. As continuous stimulation proceeded, the action on the uterus slowly disappeared so that after a short time up to 70% of the total active material secreted was noradrenalin.

Goldenberg and his co-workers (1949) have also applied chemical and biological methods to show the presence of noradrenalin in commercial extracts of adrenal medulla from cattle and found that these contained 12–18% noradrenalin, and one sample contained as much as 36%.

Tullar (1949) and Bergstrom *et al.* isolated and identified levo-noradrenalin from such extracts. Bülbring (1949) showed that suspensions of ground dogs' and cats' suprarenals are capable, during 1 hr. incubation at 37°C., of converting noradrenalin to adrenalin. Bülbring and Burn (1949c) proved that noradrenalin is methylated to form adrenalin in the course of perfusion of the dog's suprarenals with heparinized blood from a pump.

For many years, the racemic form of noradrenalin resisted resolution, and it was not until 1948 that the workers at the Sterling-Winthrop organisation (Tainter, Tullar, and Luduena) succeeded in achieving this by taking advantage of the fact that only the L-isomer forms a hydrated diastereoisomer with D-tartaric acid. The very active levo-noradrenaline is now available commercially.

For its determination, some tests similar to those described under adrenalin are usually carried out. The most useful for deciding whether the activity of an extract is due to noradrenalin or adrenalin are those where the ratio of equiactive amounts is high. In the isolated non-pregnant rat's uterus (Test 9), for example, the ratio lies between 30 and 100, adrenalin being far more active than the corresponding isomer of noradrenaline (West, 1947b; Tainter *et al.*, 1948; Luduena *et al.*, 1949). In fact, the test on the rat's uterus is the most sensitive and specific of the known tests for adrenalin, and has been used by Gaddum *et al.* (1949) to estimate adrenalin in a mixture of the two drugs.

Most workers use the rat uterus and perfused frog heart (Test 5) methods, where the ratio of equiactive amounts of noradrenalin to adrenalin are 30–100 and 20 respectively, to give the adrenalin value, and the rabbit duodenum or ileum (Test 2) and cat blood pressure (Test 1) methods, where values near to unity are recorded, to give the sum of adrenalin and noradrenalin concentrations. Then by difference, the noradrenalin value can be found. The test on the rat's colon stimulated by acetylcholine in a manner similar to that used for the non-pregnant uterus of the rat has given ratio values below 1.0, showing that on this tissue noradrenalin is more active than adrenalin. In fact, this is the most sensitive and specific test for noradrenalin.

Bülbring and Burn (1949b) described a method of estimating noradrenalin by using the fact that the denervated nictitating membrane of the cat becomes relatively much more sensitive to noradrenalin than the normal membrane which is more sensitive to adrenalin. Thus the ratio of the size of contraction by the denervated membrane to that by the normal membrane is greater the larger the proportion of noradrenalin in the mixture. By this means, it is possible to test the activity of an extract in a spinal cat not only by the effect on the blood pressure, com-

paring it with adrenalin, but also on the nictitating membranes by comparing it with an equipressor dose of a known mixture of adrenalin and noradrenalin. Denervation of the nictitating membrane by removal of the superior cervical ganglion took place under a sterile operation with ether anesthesia 7 to 10 days before use.

Gaddum *et al.* (1949) have used the denervated nictitating membrane of the cat, further sensitized by intravenous cocaine (8 mg./kg.), as a sensitive test for adrenalin or noradrenalin (5×10^{-8}). Injections are made through a special T-shaped cannula in the carotid artery. The two short horizontal arms are so shaped that they can be tied into the central and peripheral ends of the artery. The third arm is expanded to form a small reservoir containing air and closed by a rubber cap through which injections are made. An anticoagulant is used. This method has the advantage that control cat or rabbit plasma has little or no effect on the membrane in doses of 0.5 ml. injected rapidly, so that the concentrations of the amines in test plasma can be directly measured.

In their classical study of the relation between chemical structure of amines and their sympathomimetic actions, Barger and Dale (1910) noted that a dose of ergotoxine sufficient to reverse the pressor effect of DL-adrenalin in the spinal cat did not reverse that of DL-noradrenalin. Other adrenalin antagonists such as yohimbine, certain dioxaine derivatives and dibenamine have been used to demonstrate this difference of the pressor effect of the two levo-isomers. But this is not always true, and West (1949) has shown that it is possible to obtain the vasodepressor action after ergotoxine and dibenamine by using larger doses of noradrenalin, but the mechanism by which this response is produced is not clear.

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Posterior Pituitary Lobe, Hormones

By R. H. THORP

CONTENTS

I. Introduction

It is uncertain, even today, whether the posterior lobe of the pituitary gland produces one or more hormones, but when extracts of the lobe are injected into animals three distinct actions can be seen. Smooth muscle is stimulated to contraction, an effect particularly noticeable on the uterus in the female; there is peripheral vasoconstriction with a resultant pressor action and an antidiuretic action as the result of enhanced water reabsorption by the renal tubules. These effects have been separately produced by isolated fractions of such an extract although there is always some measure of the other actions shown by the crude extract alone. Much of the fundamental work on the posterior pituitary lobe has been done by Kamm *et al.* (1928), and these workers were able to prepare fractions which showed the oxytocic and pressor activities

nearly completely separated. In their view there are, therefore, at least two substances present in crude extracts, and this is supported by the work of Heller (1939) who has put forward evidence that the pressor and antidiuretic actions, which were common to Kamm's pressor fraction can themselves be separated. The latter worker holds the view that there are at least three separable fractions in the simple extract. A contrary opinion is held by Abel (1930) who believes that there is a single chemical entity which is easily degraded into simpler substances which show the individual properties just described. This view is largely based on the isolation of a very potent preparation (Abel *et al.*, 1923) capable of stimulating the uterus in a dilution of 1 part in 2×10^{10} and which exhibited oxytocic and pressor properties in practically the same proportions as the original extracts. The two preparations of oxytocin and vasopressin of Kamm (1928), on the other hand, when mixed in the appropriate proportions also produce the effects of the whole posterior pituitary extract.

Very little is known of the chemical composition of the active constituents of the posterior pituitary hormones, but Stehle and Fraser (1935) believe them to be polypeptides of high molecular weight, and Stehle and Trister (1939) have identified several of the amino acids present by hydrolysis of relatively pure extracts.

The stability of posterior pituitary extracts is high compared with most other hormones, and in acid solution the activity is not significantly reduced by boiling or autoclaving for periods of half an hour or less, but the activity is lost if such preparations are administered by mouth, due to digestion by the gastric enzymes.

II. Standard Preparation

1. International Standard

In order to achieve uniformity, an international standard was set up for posterior pituitary lobe extracts in 1925 from a powder prepared by Smith and McClosky (1924), but at this time any worker was permitted to prepare a similar powder using the methods described by these workers and to regard such a preparation as equivalent to the international standard.

In 1935 it was decided by the Commission on Biological Standardisation of the Health Organisation of the League of Nations to have only one international standard and the material is now held at national centers in the various countries concerned. The international standard has an activity of 2000 units/g. and the unit is defined as the activity of 0.5 mg. of this material both for oxytocic and pressor assays.

2. Preparation of Subsidiary Standard

It is sometimes convenient to prepare material for use as a subsidiary standard, particularly in a laboratory concerned with the large-scale preparation of extracts of posterior pituitary lobe for clinical use. The following method can conveniently be employed.

The pituitary glands should be collected as freshly as possible at a slaughter house and frozen in a jar surrounded by solid carbon dioxide. Upon arrival in the laboratory the posterior lobes are dissected from the remainder of the gland. This is not very easily done unless the glands are allowed to thaw a little as there is an optimum hardness at which the removal is most readily accomplished. The posterior lobes are then placed in cold acetone using about 4 ml. for each lobe and keeping the container surrounded by refrigerant. After about 3 hr. the lobes can be cut up and transferred to a similar volume of fresh acetone and left overnight. The acetone is then poured off and the glandular material dried in a dessicator over phosphorus pentoxide. The dried material is next powdered to the size of a No. 40 mesh sieve and further dried in the dessicator. Fat removal is next completed by extraction with pure acetone in a Soxhlet extractor for 3 hr. and after drying, the powder may be stored in a dessicator or in sealed tubes at a low temperature. After adequate assays in comparison with the international standard preparation, such material may be adopted as a working standard for the particular laboratory. It is unlikely to be as potent as the international standard but the activity of 1200 to 1600 units/g. is typical of powders prepared in this way.

3. Preparation of Standard Extracts

In order to use the standard preparations an extract must be prepared by weighing out a portion of the solid of approximately 10 mg. in a stoppered weighing bottle and taking great care to avoid undue exposure of the powder to air in view of its hygroscopic nature (Gaddum, 1927). The weighed sample of the powder is then transferred quantitatively to a hard-glass boiling tube with the aid of 0.25% acetic acid and the volume adjusted to 1 ml. for each milligram of the powder. The tube is plugged with cotton wool and placed in a boiling water bath for 2 min., cooled, and the contents filtered through a small filter paper. The filtrate contains an activity of 2 units/ml. It is essential to prepare the standard extract in exactly this way since slight variations produce differences which can be detected in differential assays of the component activities.

III. Methods of Assay

The potency of the crude extract of posterior pituitary lobe can be assayed by means of any of the three characteristic properties described previously, and although a differential analysis would be essential to follow processes of purification it is usual to assay simple extracts by means of the oxytocic action alone, especially as this is the action most frequently employed in therapeutics.

The methods of assay most commonly employed are the guinea pig uterus assay and the chicken blood pressure method for oxytocic activity, the spinal cat and the anesthetized dog methods for pressor activity, and the delay in water diuresis in the rat for the antidiuretic activity. There are various more recent methods and modifications of the standard ones which are described in proximity to the description of the relevant usual methods.

1. Methods for the Determination of Oxytocic Activity

A. THE GUINEA PIG UTERUS METHOD

The method employing the guinea pig uterine muscle is the oldest and most frequently practiced method for the assay of oxytocic activity, and has the added attraction that it utilizes the action for which posterior pituitary preparations are most commonly employed. This method was first described by Dale and Laidlaw (1912) and has been extensively modified with the passage of time.

Fundamentally the assay comprises a comparison of the action of the test and standard preparations upon guinea pig uterine muscle maintained in Ringer's solution and arranged to record quantitatively changes in length upon a kymograph. Technically the method is rather difficult and does not normally give a measure of the fiducial limits of the estimate of potency obtained.

A convenient form of apparatus is shown in Fig. 1 and consists of a water bath, which can with advantage be constructed of sheet Perspex as illustrated, surrounding two inner vessels. The temperature of the water bath is kept constant by means of a suitable electric heater and thermo-regulator, and the water is stirred by aeration. In the apparatus illustrated a 250-w. electric immersion heater is fitted and is of a type permitting easy replacement of the heating element without emptying the bath. The regulator is a bimetal thermo-regulator capable of switching currents up to 2.0 amp. without the use of a relay on an alternating-current supply; with direct current a relay would be essential. The inner vessels are used to hold the uterine segments and are of 20 ml. capacity. They can be filled with Ringer's solution from the two jets

controlled by glass stopcocks and connected to a glass coil leading from a reservoir of solution about 3 ft. above the bath. The glass coil has a capacity of 200 ml. so that there is no possibility of a temperature change when fresh Ringer's solution is added. The inner vessels are held in place in rubber stoppers and are emptied by tubes at the bottom and spring clips on short rubber connections. The lower end of the segment of uterus is hooked over a sharp prong on a glass tube through which compressed air is passed to oxygenate the surrounding Ringer's solution, and the upper end is fixed by means of a bent glass hook to the thread attached to the frontal writing levers seen in Fig. 1. The glass tubes are held rigidly by the two clamps and thumb screws seen in the figure, so that the uterus and hook are completely submerged in the solution. The smoked drum can be adjusted to give a surface speed of 0.1–0.5 cm./min.; the author favors the slower speed.

The composition of the Ringer's solution used for the preparation influences the validity of the estimate obtained, by altering the response of the uterus to differing ratios of oxytocic and pressor activity, and the formula given below is most generally suitable.

Sodium chloride (NaCl)	9.00 g.
Potassium chloride (KCl)	0.42 g.
Calcium chloride ($CaCl_2$)	0.24 g.
Magnesium chloride ($MgCl_2$)	0.0025 g.
Sodium bicarbonate ($NaHCO_3$)	0.5 g.
Dextrose	0.5 g.
Water	1.0 l.

The distilled water used in the preparation of this Ringer's solution must be quite free from copper, which is exceedingly toxic to the uterine muscle, and it is best to use water condensed in glass still, although a tinned iron condensing tube is used in several laboratories in England and produces water of excellent quality for this purpose. All the salts used in the preparation of the Ringer's solution must be of analytical grade; the substance causing most frequent difficulties is the sodium chloride. The author has found only certain products to be sufficiently pure for this work, although all samples tried were A. R. quality. This is supported by the work of Pittenger and Quici (1923) who showed that there was no correlation between batch analysis and suitability, but that all large crystal batches proved satisfactory and were presumably the first crops of the crystals obtained in the manufacture and therefore less likely to be slightly contaminated.

The guinea pigs used for this test are the least satisfactory component of the assay and do not all give uteri which respond in a regular

manner under the influence of posterior pituitary extract.   It is essential
to use guinea pigs which have been separated from males at weaning
and maintained separately until required.   A satisfactory weight range
is 150–250 g., and it is essential that only animals in diestrus be used.
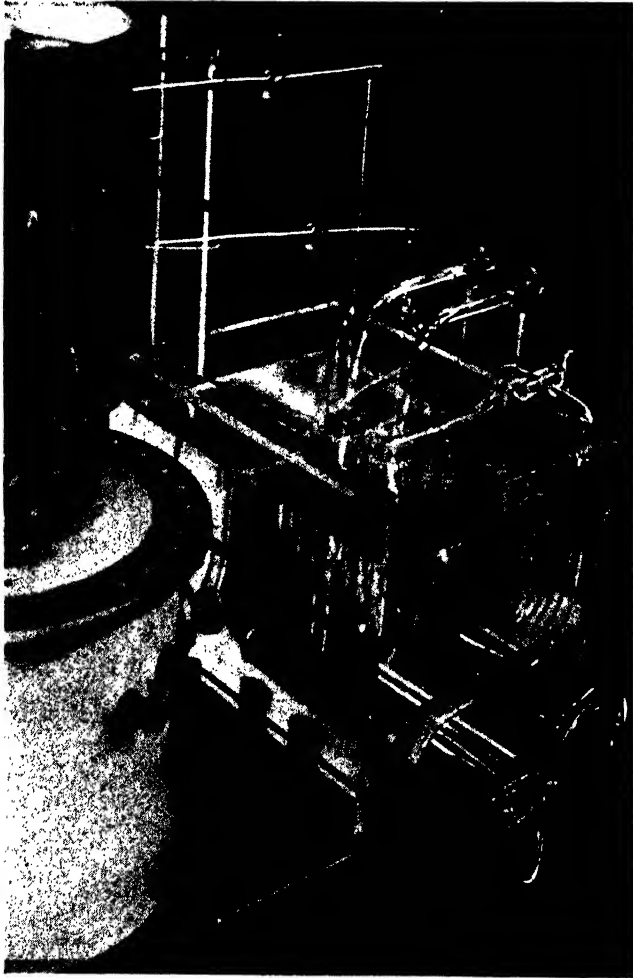
FIG. 1.   General arrangement of isolated organ bath for use in the guinea pig uterus
assay of oxytocic activity.

In this state the vaginal aperture is closed with epithelium, and the pigs
may safely be employed.   Estrogen-stimulated uteri develop spon-
taneous rhythmic contractions whereas the uterus taken in the diestrus
state shows practically no fluctuation of the baseline when in a relaxed
state in the bath.

To set up the preparation, a guinea pig is killed and allowed to bleed out, and the uterus is dissected. The two horns of the uterus are freed and separated by cutting across the junction of the horns. In the apparatus illustrated the two horns are used together, but one can be used first if only a single bath is available and the second stored in Ringer's solution in a refrigerator for a few hours or even overnight. The glass spike is pushed through the uterus and the ovary is hooked up on a hook at the end of the thread. The writing lever is then adjusted to give approximately a twofold magnification of the uterine contraction so that the height of the record on the kymograph paper is approximately 5–6 cm. In order to obtain satisfactory records a light aluminium lever should be used, and it may be necessary to counterbalance some of the tension on the uterus by means of a small sliding weight of approximately 0.2 g. In some cases a better response may be obtained by increasing the tension upon the uterus, by placing the slide near the writing point. Doses are added to the bath by means of a calibrated 1-ml. syringe according to a set schedule of times. Upon the addition of a dose, 5 min. or more, if the contraction is not stabilized, is allowed before the bath is drained out and filled with fresh Ringer's solution. A further 15 min. should then elapse before another dose of pituitary extract is added. In order to eliminate the possibility of a variable interaction between subsequent doses, such a timing system must be rigidly adhered to in any one experiment.

Various technics have been described for the comparison of the standard and unknown solutions, but it is first necessary to find a dose of the standard extract of posterior pituitary lobe which will cause a suitable submaximal contraction. A suitable dose for preliminary trial is 0.6 ml. of a $\frac{1}{100}$ dilution of the 2 units/ml. extract of standard posterior pituitary lobe powder in 0.9% saline. If this produces a satisfactory response, the dose is increased to 0.8 ml., to show that the contraction with the smaller dose was not maximal. If the first dose produced a maximal effect, it may be necessary to dilute the extract still further, or to use a smaller dose. The aim should be to determine the dose which produces a contraction of approximately 75% of the height of the maximal contraction. When this dose has been found, it is possible to proceed with the assay.

The object of the test is to determine the ratio of doses of test sample and standard producing the same height of contraction, but the uterus may show a gradual change in sensitivity so that each subsequent dose produces proportionately rather less effect. A satisfactory experimental design which helps to eliminate this effect is to put in a dose of the standard which gives a satisfactory response and then a second dose

giving either a greater or smaller response, varying from the first by an increment of approximately 20%. Two identical successive doses of the test sample, judged to produce a response intermediate between those from the two doses of standard, are then put into the bath. Finally the 2 doses of standard are repeated in reverse order and again should give 2 responses larger and smaller than those of the test material. Such an experiment is shown in Fig. 2 and can be taken as showing that the quantity of the oxytocic hormone in the test dose was intermediate between the two standard amounts.
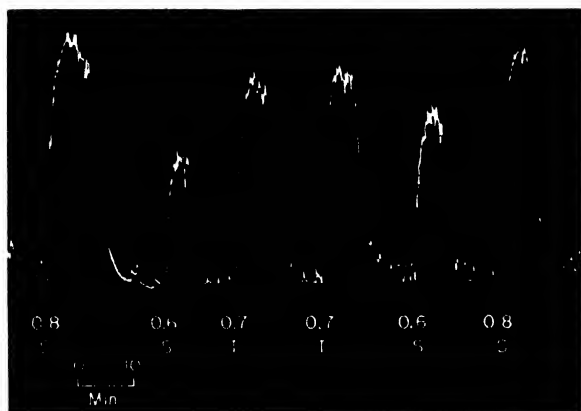
FIG. 2. Tracing obtained in a typical assay of oxytocic activity by the guinea pig uterus method. S = standard extract diluted 1/160; T = a test sample diluted to produce a comparable response to that of the standard. In this case 0.7 ml. of the sample produces an effect intermediate between that caused by 0.6 and 0.8 ml. of the standard.

A test must normally be preceded by several approximate comparisons of the test sample and the standard, unless the potency of the former is reasonably well known. When a series of 3 such tests has been performed satisfactorily, it is reasonably established that the standard error of the mean potency value obtained is in the region of ± 10%. For normal routine purposes 3 such tests are considered satisfactory, but for such purposes as the setting up of a subsidiary standard, many more tests should be performed.

The guinea pig uterus assay is often further complicated by rhythm, which means that if the same dose of preparation is repeated the contractions may be alternately large and small. Under these circumstances the tissue cannot be used satisfactorily, and a fresh animal must be used.

B. MODIFICATIONS OF THE UTERUS METHOD

There are many factors influencing the accuracy of the guinea pig uterus method, and a great many workers have investigated certain aspects of this subject.   The effect of the constituents of Ringer's solution has been the subject of much published work, particularly with a view to finding a physiological solution which minimizes the rhythm mentioned above.   The salts of the Ringer's solution which have been most studied are calcium and magnesium.   The most usual calcium content is 0.24 g./l. of anhydrous calcium chloride.   Variations in this figure have been examined by Kochman (1921) who recommended a smaller concentration on the ground that less spontaneous contraction of the uterus muscle was observed.   The view is generally accepted that the occurrence of rhythm is largely due to the addition of hormone to the bath, at different phases in the spontaneous basal contractions of the muscle, and that reduction of the latter to produce a more steady baseline is also associated with reduction of rhythm.   More recently de Jalon (1947) has shown that reducing the calcium content to 0.12 g./l. accelerates the contractions of the muscle in the presence of the oxytocic hormone and also permits it to recover more quickly between doses.   The general view of workers in this field, however, indicates that the concentration of calcium originally stated is a very suitable one.

The evidence for the importance of magnesium is much less clear. In an early description of Burn and Dale (1922) a concentration of 0.005 g./l. of magnesium chloride was used, and they stated that this small amount tended to suppress the irregular spontaneous contractions of the muscle.   In a later description Burn (1937) doubted whether the addition of magnesium was of any real help.   The effect of magnesium has recently been reinvestigated by de Jalon (1947) and Hsu (1948). Using amounts of magnesium up to 0.29 g./l. of magnesium chloride, de Jalon found that the relation between the dose of pituitary extract and the uterine contraction was enhanced, so that the discriminating power of the uterus was improved.   Hsu has continued this work and has shown that 0.45 g./l. of magnesium chloride produces a very satisfactory Ringer's solution from the point of view of increasing the sensitivity of the uterine muscle to small doses of pituitary extract and preventing spontaneous alternating rhythms.   It is also pointed out in this work that the same solution depresses the response of the uterus to histamine, and hence it is advisable to substitute the normal Ringer's solution when the presence of histamine needs to be demonstrated.

These workers have been concerned solely with the establishment of suitable conditions to give a workable assay for the oxytocic principle but have not described the effect of the different constituents of the

Ringer's solution on the result obtained in the presence of the pressor principle (vasopressin) in various amounts, but Fraser (1939) has shown—using a modified Tyrode's solution described by Van Dyke and Hastings (1928)—that vasopressin exerts a considerable degree of oxytocic action which was not shown in the magnesium-free Ringer's solution. He has reported that as the magnesium concentration is increased the oxytocic activity due to vasopressin increases until it eventually may equal that of oxytocin. Fraser concluded that the degree of oxytocic action ascribed to a preparation as the result of an assay depends upon the amount of magnesium added to the physiological saline solution, when the ratio of the oxytocic and pressor activities differs from that of the standard preparation. This problem has been studied exhaustively by Stewart (1949a), since it greatly affects the results obtained in the oxytocic assay of dried pituitary lobe powders in different commercial laboratories. Some of the results he obtained are reproduced in Table I,

TABLE I

Extraction of Commercial Samples of Posterior Pituitary Lobe Powder under Different Conditions of Manufacture

(Stewart, 1949)

Treatment	Pressor activity U./g.	Oxytocic activity[a] U./g. ± Std. error.	Ratio Oxytocin Vasopressin
A	888	883 ± 56	1.00
B	552	677 ± 52	1.22
C	482	196 ± 14	0.41
D	658	508 ± 21	0.77

[a] Assayed by the chicken depressor method of Coon.

which shows that the ratio of oxytocic to pressor activity varies from one batch of material to another and is certainly not always the same as that of the standard preparation. Stewart does not state the standard error of the pressor determination, but used the anesthetized dog method, described later in the chapter, for which an accuracy of $\pm 10\%$ was claimed by the original workers. The magnitude of the errors which variations in the Ringer's solution can introduce is well shown in Tables II and III, taken from Stewart's paper. Table II shows the changes in apparent oxytocic activity of purified samples of oxytocin and vasopressin. (Pitocin and Pitressin, Parke Davis & Co.) in the presence of added magnesium. It will be seen that the enhanced oxytocic action is much more predominant for vasopressin than for oxytocin, in agreement with the work of Fraser (1939). Table III shows the effects which can be obtained in practice using mixtures of varying oxytocin to vasopressin ratio (prepared from Pitocin and Pitressin mixed in various proportions) but having known oxytocin content, when two different Ringer's solutions

are employed.   This again shows that the addition of magnesium introduces the greatest errors when there is a greater proportion of vasopressor activity in the sample than in the standard extract.

TABLE II

The Influence of Excess Magnesium Chloride on the Response of the Guinea Pig Uterus to Pitocin and Pitressin—Basal Concentration of Magnesium Chloride in Ringer's Solution = 0.00025 %

(Stewart, 1949)

Excess conc. of magnesium chloride %	No. of uteri	Increase in response	
		Per cent	Std. error %
	Pitocin		
0.00125	3	5	2.1
0.0025	4	43	4.4
0.005	2	82	2.2
0.01	6	70	26.6
0.025	4	45	24.8
0.05	8	46	24.8
0.1	2	187	3.0
	Pitressin		
0.00125	1	Nil	
0.0025	2	129	9.0
0.005	3	104	10.6
0.01	2	44	10.0
0.025	3	59	18.1
0.05	4	95	30.5
0.1	2	76	2.0

TABLE III

Typical Differences in Guinea Pig Uterine Assay Results Using Two Usual Formulas for the Physiological Salt Solution

(Stewart, 1949)

Ratio Pitocin/Pitressin	Actual total oxytocic potency. U./ml.[a]	Ringer's soln. (B.P. 1948) potency found. U./ml.	Ringer's soln. with added mg. (Hsu, 1948)[b] U./ml.
4.00	10.1	8.0	10.6
2.00	10.2	9.2	10.6
1.00	10.4	10.0	10.6
0.50	10.8	9.3	13.6
0.25	11.6	11.2	13.6
0.125	13.2	13.0	19.2

[a] Allowance has been made for 4 % oxytocic hormone impurity in Pitressin and for 4 % pressor hormone impurity in Pitocin in computing the actual total oxytocic potencies of the solutions.

[b] Hsu (1948) Ringer's solution contains 0.045 % MgCl₂.

Varying concentrations of calcium also influence the response of the uterus differentially to the oxytocic and pressor principles, but Stewart was unable to produce definite contractions of the uterus in

the absence of calcium with either fraction.   Figure 3 shows the tracing
he obtained with the purified pressor fraction using Ringer's solution
without calcium and the effect of the addition of small amounts of
calcium chloride to the bath prior to the addition of the hormone.   He
showed that with such small excesses of calcium chloride as 0.005% to
0.01% the oxytocic effect was potentiated whereas with amount of 0.01%
to 0.025% in excess the effect due to the pressor component was greater.
With a much greater excess of calcium it was, again, the oxytocic hormone
which showed the greatest degree of potentiation.
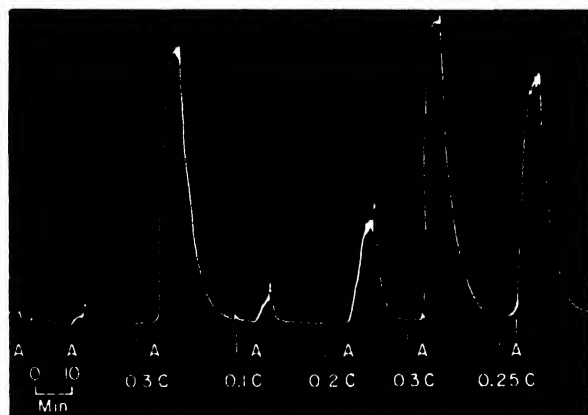
Fig. 3.   The influence of the addition of calcium chloride to Ringer's solution
containing no calcium chloride on the response of the isolated guinea pig uterus to
Pitressin. A = 0.8 ml. of Pitressin, 20 pressor units/ml. diluted 1/120; C = dose
in milligrams of calcium chloride.   (Stewart, 1949.)

The effect of temperature of the uterine muscle upon the sensitivity
to oxytocin has been studied by Hamburger (1946), who showed that the
sensitivity to oxytocin is maximal at 32°C. but that assays could be per-
formed between 25°C. and 42°C., and he considered that the best tem-
perature, for general use, was 28°C. because there is good sensitivity
at that temperature together with a short reaction time.   Hamburger
stressed the importance of the constancy of the temperature of the bath
throughout any one experiment since a change in temperature of 1°C.
between 33°C. and 42°C. caused a change in sensitivity of 14%.   The
slopes of the regression lines obtained by plotting the dose of the oxytocic
principle against the height of contraction were not significantly different
at 32°C. or 37°C. so that no increase in accuracy was to be obtained
at the temperature of the greatest activity.

C. STATISTICAL EVALUATION OF RESULTS

The technic of the arrangement of the doses and the application of statistical analysis to the guinea pig uterus assay has been investigated by several workers. Burn (1937) describes the use of a series of 4 doses, 2 of which are the standard preparation, A, and 2 the sample, B, given in the order A, B, B, A arranged to test equality or inequality of the two materials. Tests on this system are then repeated until the potency has been adequately demonstrated. Hamburger (1945) considered that it was better to obtain a large number of contractions with several doses, ranging from minimal to maximal in effect and to plot the dose-response curve from the mean values of the contraction obtained for each concentration. The latter method, although very desirable, requires that the uterine muscle should behave well over a large period of time, so that at least 25 responses can be obtained in any one experiment.

Morrell (1940) and his co-workers described a technic in which each uterine horn was cut into 4 segments, and the 8 pieces thus obtained, attached to 8 levers, suspended in a large bath. Doses of pituitary extract were used which caused only some of the 8 strips to contract, and the behavior of each strip was treated as a quantal response. Doses of the standard and test preparation were alternated and arranged to cause in the first part of the experiment, contraction in about 50% of the strips, and later in 25% of the strips. The results were then calculated by the normal dose response methods for a quantal assay as described by Gaddum (1933) and Bliss (1935a, 1935b). A determination of the slope of the regression line was obtained by this procedure, and this was shown to vary significantly from one test to another, so that it was considered essential to use a standard preparation in each assay. This method enabled statistical weights to be assigned to the potency value determined in any assay, but has the principal disadvantage that a quantal method has replaced a method producing a continuous variate, and hence the authors (Bachinsky *et al.*, 1945) described a modification using a continuous response by measuring the height of the response of each strip of uterus, in the experimental arrangement described above. In this case, again, a 2-dose arrangement was employed for both the sample and the standard preparation, and integral determination of the slope was included. In a series of 23 assays, by this method, the standard error of the mean potency was less than 5% in all but 5 cases, but in 10 cases, unfortunately, the true potency figure was outside the range of the limits of error of the assay result.

Some form of arrangement similar to that described first in this chapter or the method of Burn referred to above is probably a good

method of conducting the assay, in view of the difficulty of obtaining large numbers of repeatable responses. The standard error of this method was examined by Gaddum (1938) and shown to be approximately 7.7% for a satisfactory test, which agrees well with the ($P = 0.99$) limits of $\pm 20\%$, suggested in the British Pharmacopoeia, 1948, and the United States Pharmacopoeia (XIII revision).

D. OTHER METHODS FOR THE ASSAY OF OXYTOCIC ACTIVITY

Several other methods have been described for the assay of oxytocic activity and these fall into two classes: those in which the test preparation is uterine muscle and those in which a different response is used for the quantitative assessment but which is shown to parallel the oxytocic activity. Trendelenburg (1928) investigated the possibility of using the uterus obtained from freshly killed sheep for an oxytocic assay. He used 3-cm. strips of uterus, suspended in Tyrode's solution, normally taking two strips from adjacent parts of the uterus. This work showed that the sheep uterus preparation was very variable in its response to posterior pituitary extracts, many preparations being uniformly insensitive, and others sensitive at first but gradually becoming less so. In good preparations the sensitivity is about the same as that for the guinea pig, and the method can give results with a standard error of approximately $\pm 10\%$.

A modification of the uterine method has recently been described by Holton (1948) using uteri from rats. One horn of the uterus was suspended in a 10-ml. bath of Locke's solution at 32°C., modified by reduction of the calcium and glucose respectively to a quarter and a half the normal content. The lever used gave 4 times the magnification and loaded the uterus to approximately 1.2 g. The bath was aerated with 5% carbon dioxide in oxygen, although this might not be necessary when the solution is replaced at short intervals. Using non-pregnant albino rats of 120–200 g., Holton found that, out of 9 rats, 6 gave good responses. The doses of posterior lobe extract were given at intervals of 3 or 4 min. and 2 doses were found, such that the contraction for the higher dose was at least twice as great as that of the lower. The ratio usually employed for these 2 doses was 4/3, but only contractions below 80% of the maximal could be employed in order to ensure a linear relationship between response and log dose. Little trouble was experienced due to spontaneous contractions, but when these did occur they were often overcome by reducing the temperature of the bath, or the interval between successive doses.

The arrangement of the doses for the assay was due to Schild (1942) and originally described for histamine assays. Two doses of the standard

and two of the test preparation are employed, with the same ratio of high to low doses in each case.   In this arrangement each dose is given once in a group of 4 doses and the sequence of the 4 doses is determined by chance.   One of Holton's tracings is reproduced in Fig. 4 and shows the results obtained in one assay.   This method enables an estimate of potency to be obtained with an integral determination of the slope of
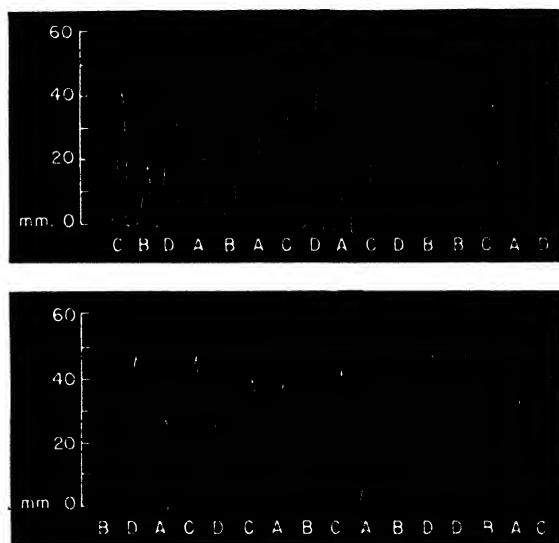
Fig. 4.   An assay of posterior pituitary extract.   The record shows 32 contractions of a rat's uterus.   The contractions are responses to 4 different doses of posterior pituitary extract A, B, C, and D, each of which is given once in each group of 4 contractions.   There are 8 groups in all.   A = 0.05 units, B = 0.04 units, C = 0.064 units, and D = 0.08 units.   B and C were treated as "standard."   A and D were treated as "unknown."   A/D = B/C = 5/8.   Estimate of unknown/standard = 1.25.   True value unknown/standard = 1.25.   A dose was put into the bath every 4 min. and washed out after 45 sec.   Weight of rat 140 g.   Temperature 34°–36°C. Load on uterus 1.3 g.   Total experimental time 4 hr.   (Holton, 1948.)

the dose-response curve.   The error of the assay is calculated by variance analysis, and the slopes of the dose-response curves for the standard and test preparations are tested for concordance.   The calculated standard error for a series of 8 assays, using this method, was 2.84%, approximately half the value found by Gaddum for the guinea pig uterine assay.

The method which Holton has described appears to be superior in precision to the time-honored use of the guinea pig uterus.   It has not yet been shown, however, that different extracts containing different proportions of oxytocin and vasopressin will give the same value by both

methods, in fact it is not known whether vasopressin will cause contractions of the rat uterus, or to what extent the oxytocic response will be influenced by magnesium ion concentration. For research purposes, under constant conditions, the method might well be one of choice.

E. THE CHICKEN DEPRESSOR METHOD

Of the methods for oxytocic activity other than by uterine contraction the principal research has been upon the utilization of the transient fall of blood pressure which oxytocin produces upon injection into birds. This phenomenon was first observed by Paton and Watson (1912) after the injection of pituitary extracts and was attributed principally to oxytocin by Gaddum (1928). The development of an assay method on this basis was due to Coon (1939); the method produces results very close to those of the guinea pig uterus method and is quicker and technically more simple. Coon described the following technic which has proved very satisfactory in the author's laboratory.*

White Leghorn chickens are used weighing 1.8 to 2.2 kg., although owing to difficulties of supply, the author has used Light Sussex birds of a similar weight satisfactorily, whereas Rhode Island Reds proved of little use. The bird is anesthetized by intravenous injection of 200 mg./kg. of sodium phenobarbital via the brachial vein and arranged for recording blood pressure from the ischiadic artery. The ischiadic artery is exposed by removing the feathers from the outer surface of the left thigh, an incision 7–8 cm. long is made in the skin, parallel to and about 1.5 cm. below the femur, exposing the gluteus primus muscle. The lower edge of this incision is retracted to expose the edge of the gluteus primus muscle overlying the semitendinosus muscle. This edge is then freed for the length of the incision, and when the free edge is lifted, the ischiadic artery, ischiadic vein, and crural vein can be seen lying along the edge of semitendinosus muscle. The gluteus primus muscle is cut at right angles near the proximal end of the incision and the resulting flap deflected and secured to the upper thigh. Lengths of the ischiadic artery and crural vein are dissected free and the artery cannulated.

For recording blood pressure a mercury manometer may be used with an inside diameter of 2.5–3 mm. using a hollow ebonite float to operate the recorder pointer, as anticoagulant a 5%–8.5% solution of sodium citrate is employed to fill the manometer. The blood pressure recorded should be approximately 105 mm. Hg and will quickly settle down to a constant level. It is convenient to use a slow-moving recording drum

* At this time the Wellcome Biological Assay Laboratory, Dartford, Kent.

with a surface speed of approximately 1 cm./min., since injections are made every 3–5 min. Injections are made directly into the crural vein by means of a 1-ml. tuberculin syringe and fine needle, using the same puncture hole for subsequent injections, covering it with a pledget of absorbent cotton between each pair of injections. The standard posterior pituitary extract (2 units/ml.) is diluted 1 in 10 with 0.9% saline and such injections made that a graded fall in blood pressure results from graded doses. The illustration of Fig. 5 from Coon's paper shows
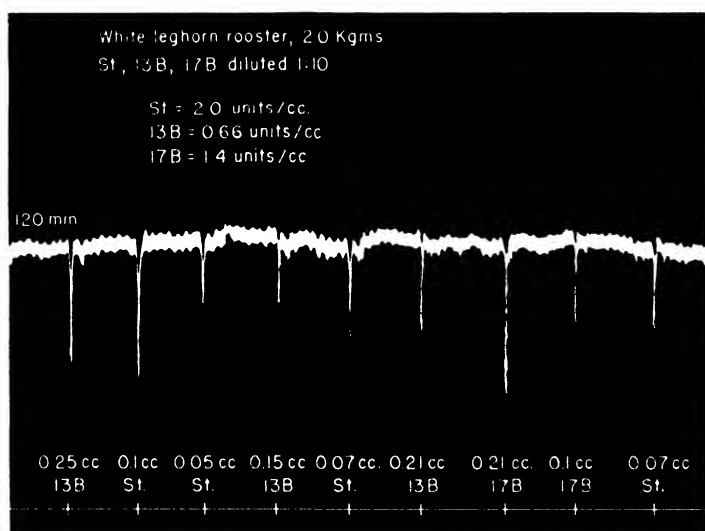
Fig. 5. A typical tracing of the assay of two unknown samples of posterior pituitary extract by the chicken depressor method. The injections were given at intervals of 3–4 min. (Coon, 1939.)

the effects observed in a good preparation. The fall in blood pressure should be between 20 and 40 mm. Hg. A suitable dose for preliminary trial should be 0.2 ml. of the diluted extract. After a suitable dose has been determined, doses of the test and standard can be injected at 3–5 min. intervals according to the suitability of the bird. The doses can be injected in any suitably arranged sequence. Coon endeavored to produce matched responses and to obtain the potency of the unknown sample by direct comparison, but a suitable experimental arrangement is a random block design with two doses of the test and standard preparations as used by Holton (1948) in the rat uterine assay.

Further work on this method has been reported by Smith (1942) and Smith and Vos (1943) and most recently by Thompson (1944). Smith and Vos showed that the depressor response in the chicken increases

linearly with the logarithm of the dose of pituitary extract given, and they used a randomized arrangement similar to that referred to above. Since this arrangement eliminates the variations in sensitivity over long periods of time, but is still subject to influence by changes in sensitivity within the period of an individual group of four doses, Thompson (1944) made use of an experimental design developed by Vos (1943) for use in the assay of ergonovine upon the rabbit uterus. In this arrangement, the dose of the standard preparation is kept constant and administered alternately with three varied doses of the test sample giving responses above, below, and equivalent to that of the standard. The dose of the test preparation giving a response greater than that of the standard should not be more than twice as great as the dose producing the smallest response. This assay must be run to an accurate time schedule and an interval of 3 min. between doses is recommended unless this allows insufficient time for the blood pressure to return to normal between doses. The accuracy obtained by Smith and Vos was such that a mean error of 6.9% was obtained using "unknown" dilutions from a standard preparation and results obtained by Thompson showed even better agreement.

Coon found that the presence of large amounts of vasopressin caused a secondary rise in blood pressure, after the initial fall, but this did not introduce appreciable errors with pressor to oxytocic ratios of less than 4/1. In addition to this secondary pressor action, however, the presence of large amounts of the pressor principle was shown to produce some degree of enhancement of the oxytocic depressor response. A slightly higher apparent potency would therefore be obtained in comparison with the isolated uterus preparation. In practice it was found that ratios of pressor to oxytocic principle of less than 2.5/1 did not produce significant deviation from the uterine value. As the assay continues, the animal becomes tolerant to the depressor response, and it may be advisable to leave the bird to recover for an hour or so, if the sensitivity becomes greatly reduced. If the blood pressure falls to an undesirably low level the injection of 4–8 mg./kg. of ephedrine sulfate frequently restores the pressure to a useful level. A chicken prepared in this way can constitute a stable preparation for 6–12 hr. and may be used to assay several unknown samples, and approximately 90% of these preparations prove suitable for assay purposes.

2. Methods for the Determination of Pressor Activity

A. THE PRESSOR RESPONSE IN THE SPINAL CAT

This method is that most generally accepted for the assay of pressor activity and was described by Dale and Laidlaw (1912) and re-examined

in detail by Hogben *et al.* (1924).   The method depends upon the degree
of elevation of the blood pressure in a spinal cat upon intravenous
injection of posterior pituitary lobe extract.   It had been shown by Dale
and Laidlaw that when the injections are made at short intervals of
time the magnitude of consecutive responses to the same dose decreases
continually, so that they attempted to match the responses to standard
and test, after these had become small and relatively uniform.   Hogben
and his colleagues, however, showed that after sufficiently long intervals
the response remains similar for identical doses, and they used an interval
of 1 hr. between doses in their successful tests.   Cats weighing from 3–4
kg. are most suitable and are anesthetized with ether, the trachea is
exposed and cannulated, and both carotid arteries are then ligated.   The
cat is then turned into the prone position and the spinal cord beneath
the second cervical vertebra is dissected out and cut cleanly across.
Artificial respiration is then started and the brain is destroyed by a
probe thrust through the foramen magnum.   The foramen magnum
can be closed with a plug of plasticine and the wound in the neck sewn
roughly together.   The blood pressure is recorded from a carotid artery
and a jugular or femoral vein is cannulated for injection of the hormone.
The blood pressure is recorded by means of a mercury manometer and
during the course of an hour will settle to a steady value which is fre-
quently of the order of 50 mm. of mercury.

The doses of pituitary posterior lobe preparations can usually only
be given at hourly intervals since the preparation will otherwise become
rapidly insensitive, although some cats do recover sufficiently rapidly
for an interval of 30 min. to be possible.   The doses of the preparation
usually given are between 0.2 and 0.6 unit of preparation of approxi-
mately 1.0 units/ml., the dilutions being prepared in 0.9% saline.

The work of Hogben *et al.* shows that this preparation is most sensitive
when the dose given is approximately that required to produce half the
maximal response and is usually the dose required to produce a rise in
blood pressure of approximately 55 mm. Hg from a resting level of approx-
imately the same value.   Under these circumstances results similar to
those shown in Fig. 6 should be obtained and from which it will be seen
that differences of dose of 15% or above can be clearly distinguished.
These authors found that as the assay proceeded the discrimination
improved, and the record of Fig. 7 shows four responses taken from the
end of a series and illustrating a clear differentiation of dose differences
of 10%.

In view of the relatively small number of injections which can be
made in a reasonable time it is usually only possible to assess the potency
of the test preparation by bracketing it between known doses of the

standard extract. This is, in fact, one of the chief objections to this
method and has led to the use, particularly in America, of the anes-
thetized dog (Kamm *et al.*, 1928). This preparation is simpler and,
although rather less discriminating in the pressor response, is one which
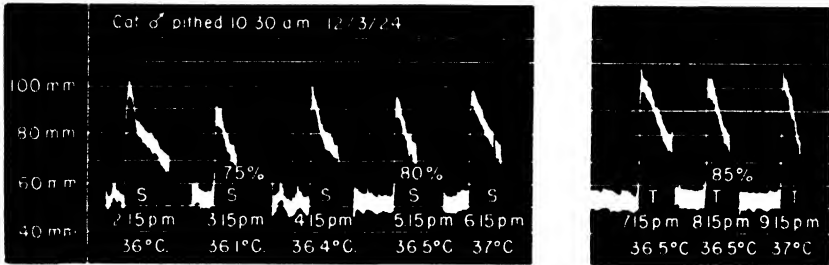
FIG. 6.   Eight consecutive injections of pituitary extract at hourly intervals on
spinal cat, showing 25, 20, and 15 %, discrimination. Slow drum (2 mm./min.).
Lined in spaces of 10 mm. pressure, lower limit 40 mm., systolic pressure about 60 mm.
throughout.   *S* and *T* are different standards of depressor free extract.   (Hogben
et al., 1924.)
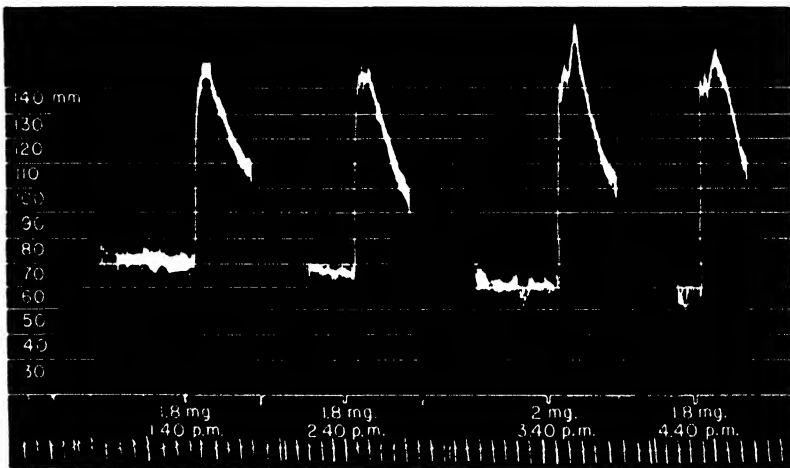
FIG. 7.   Four pressor responses to injections of posterior pituitary extract in the
spinal cat, taken at the end of a long series of responses and showing a 10 % dis-
crimination.   (Hogben *et al.*, 1924.)

can be given injections at 15-min. intervals.   It is considered by the
American workers to have an accuracy of $\pm 10\%$ and, therefore, it is
likely that the advantages more than outweigh the reduced discrimination
of the dog as compared with the spinal cat.

B. THE ANESTHETIZED DOG PREPARATION

The technic described by Kamm *et al.* (1928) used medium-sized healthy dogs deeply anesthetized with chloretone, which was suggested by the work of Rowe (1916), using a dose of 1 ml./kg. of a 40% solution of chloretone in 40% alcohol given by intraperitoneal injection. One femoral vein and one carotid artery are cannulated, the former for the injection of the test materials and the latter for recording the blood pressure. The depth of anesthesia was maintained such that only a slight conjunctival reflex remained. Doses of 1 ml. of a 1-in-25 dilution of the standard extract were injected alternately with doses of the preparation under examination. The interval between successive administrations was 15 min. These workers report that the technic depends for its success on close adherence to detail, and hence the timing is of considerable importance. They adjusted the doses of the test material to produce a response matching that of the standard and repeated the match in two series of doses in one dog, using a second dog for confirmation if necessary. This test has been used by Stewart (1949a) who reports (1949b) that it was always possible to distinguish differences of 20% in the dose of the standard preparation, and in good preparations a differentiation of 10% was possible. He considered that the simpler technic and the use of a shorter interval between doses outweighed the rather inferior discrimination shown by this method when compared with the more usual spinal cat preparation.

C. RAT PREPARATIONS FOR PRESSOR ASSAYS

In view of the expense and protracted nature of experiments of this type upon cats or dogs, attempts have been made to use the rat for this purpose, and this work has largely been described by Landgrebe *et al.* (1946). These workers used anesthetized male rats and in some cases spinal animals and found the following procedure to be the most satisfactory.

Rats weighing more than 380 g. and preferably above 450 g. should be used and are anesthetized with 0.3 ml./kg. of "Dial" liquid intraperitoneally ("Dial" Ciba, or a liquid prepared by taking 0.4 g. monoethyl urea, 0.4 g. urethane, 0.1 g. diallylbarbituric acid and making up to 1 ml. with water). The animal is kept warm and given a total of 400–500 mg. of urethane in doses of 100 mg. after an interval of 1 or 2 hr. After a further 30 min. the animal is prepared for operation on a warm table, keeping the rectal temperature at 33°C. The trachea is cannulated and the pharyngeal extremity plugged with cotton wool and the vagi and associated spinal nerves are cut. The central nervous sys-

tem is next pithed caudally from the anterior tip of the pelvic girdle to eliminate fluctuations of the blood pressure when injections are made via the femoral vein.   The femoral vein is then cannulated for injection and 1–2 mg. of heparin is injected.   One carotid artery is next ligated, and the second is cannulated and arranged for recording the blood pressure by means of a narrow bore mercury manometer, using 3.8% sodium citrate as an anticoagulant.   The preparation is next given 12–18 mg. of soluble pentobarbitone by the venous cannula, and artificial respiration is given at 40 strokes/min.   Pentobarbitone in that dosage
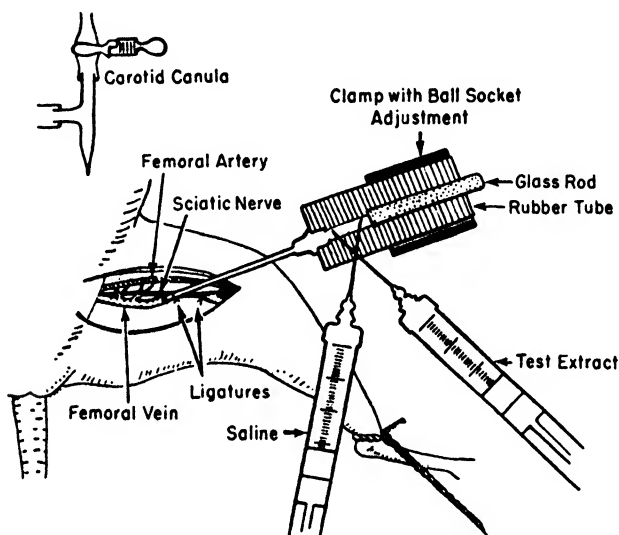
FIG. 8.   Details of apparatus and femoral cannulation.   External and internal diameters of carotid cannula tip 1.0 mm. and 0.7 mm.   (Landgrebe *et al.*, 1946.)

causes cessation of normal respiration without increasing the degree of general anesthesia or lessening the blood pressure.   The pituitary preparation is injected at 15-min. intervals via the venous cannula, and the authors suggest the arrangement illustrated in Fig. 8, which ensures that the whole of a dose of a hormone is washed into circulation.

Animals prepared in this way have a relatively steady blood pressure baseline and survive for 12–15 hr.   Figure 9 from this paper shows the pressure difference produced by 30% changes in dose, and a 10% difference is usually easily discriminated even in the less sensitive preparations. Figure 10 shows the dose-response relationship for this preparation, and it will be seen that the best discrimination occurs between 0.006–0.008 I.U. of posterior pituitary lobe extract.   When much larger doses are given, up to 0.032 I.U., irregular responses are produced but this effect

is not normally serious since it only occurs with 4–5 times the most suitable dose.

Landgrebe *et al.* state that the best experimental design is that of Burn (1937), previously described in connection with the oxytocic assay, in which doses of the standard preparation are found which cause responses above and below that of the unknown although, one again, the randomized block design would seem worthy of a trial.
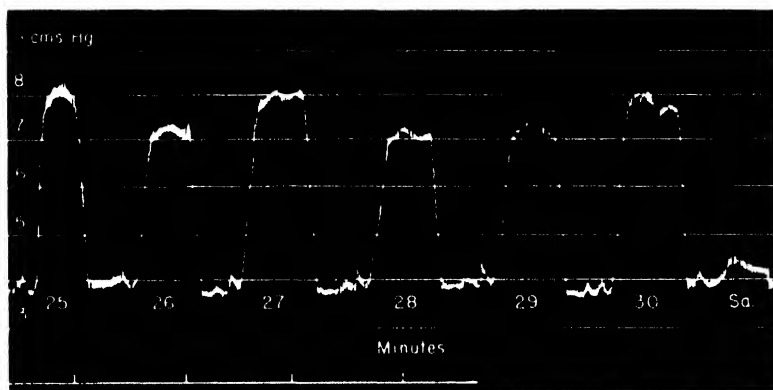
FIG. 9.   Pressor responses to injection of posterior pituitary lobe extracts using the rat preparation.

25 = 0.008 I.U. Post. pit. Ext.
26 = 0.006 I.U. Post. pit. Ext.
27 = 0.008 I.U. Post. pit. Ext.
28 = 0.006 I.U. Post. pit. Ext.
29 = 0.006 I.U. Post. pit. Ext.
30 = 0.008 I.U. Post. pit. Ext.
Sa = 0.2 ml. saline
(Landgrebe *et al.*, 1946.)

The response of the rat preparation is similar to that of the cat to histamine but the former is far less sensitive to the drug and hence the amount normally present in commercial powders is insufficient to interfere with the pressor assay.   In fact, histamine contamination up to 2% by weight in a posterior pituitary lobe powder has no effect upon the pressor assay.   The rat preparation is normally 15–20 times as sensitive as a good spinal cat and will often detect 10% and certainly discriminate 20% dose intervals, which is again, good or better than, the cat.   The shorter time interval of 15 min. is a very great advantage over the cat, and the preparations are satisfactory for about 15 injections.

Shipley and Tilden (1947) have examined the pithed rat and, using animals of 150–300 g. anesthetized with sodium amytal and completely

pithed, they found that the blood pressure settled to 40–60 mm. Hg after about ⅓ hr. These preparations were about 2–5 times as sensitive as the cat to posterior pituitary lobe pressor hormone and worked satisfactorily for 5–7 hr. Both these workers and Landgrebe and his colleagues found that 90% of their preparations were successful. It would appear that the rat methods are well worthy of use in the pressor assay of posterior lobe pituitary preparations.
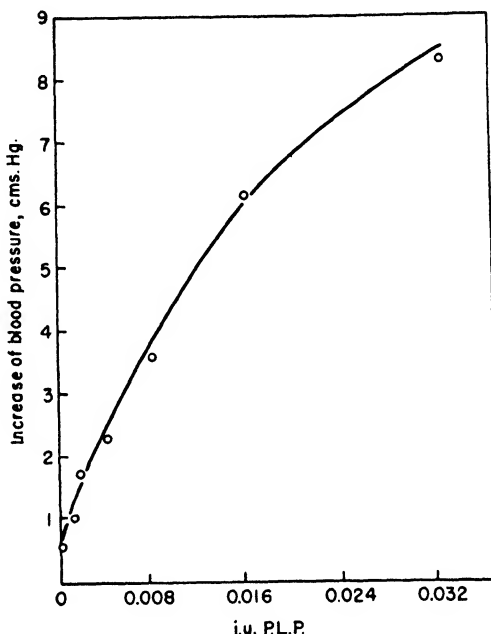
FIG. 10. Dose-response curve obtained for the pressor response in the rat using posterior pituitary lobe extract. (Landgrebe *et al.*, 1946.)

3. Methods for the Determination of Antidiuretic Activity

The first attempt to utilize the antidiuretic activity of preparations from the posterior pituitary lobe as a method of biological assay was described by Gibbs (1930), using mice. Groups of 8 mice were used for these experiments and each animal was placed in a glass funnel on a gauze mesh disk and covered with a second funnel. Beneath each was placed a graduated cylinder for the collection of the urine. Each mouse was given an injection of warm tap water intraperitoneally and then 4 of the mice were given a dose of pituitary extract by subcutaneous injection. The actual dose Gibbs used is not exactly described but was presumably 0.1 ml. of a 10 units/ml. preparation. Gibbs indicated the

flow of urine by one or more " + " signs and noted that after about 2 hr. the control mice were excreting increasing quantities of urine whereas there was a marked delay in the urinary excretion of the pituitary treated mice.

Burn (1931) followed up this work and first suggested the use of rats instead of mice since the former animals could be used in a cross-over test (Marks, 1926), and also because they produce an easily measurable volume of urine, thus converting the method from a quantal to a quantitative one.   Gibbs suggested the expression of the potency of the preparation in units based on the mouse response but Burn pointed out that any good method of biological assay must be a comparison between the action of the test preparation and that of a standard of the same material.

A. THE RAT METHOD

The technic used by Burn employed a group of 16 rats housed in gauze-floored cylindrical cages supported over large funnels with 4 rats in each cage.   For this technic the rats should all be males and weigh between 120 and 240 g. and they must be starved at least 12 hr. before the test is commenced, although free access to water is still permitted. This procedure results in a greater change in the delay in diuresis with a given incremental change in dose, than is the case with rats fed up to the time of the experiment.

The rats are weighed for the test and given 5 ml./100 g. of warm tap water by stomach tube.   The stomach tube can conveniently be made from a length of No. 3 gum-elastic catheter attached to the end of a 10-ml. syringe by forcing it over a short blunt needle of sufficient diameter to produce a tight fit.   Burn describes a simple, and now widely used, gag to assist in this operation, but the author has found that if the rat is held vertically by an assistant by the thumb and first finger, behind the ears, and the tail is held straight down, it is easily possible to insert the stomach tube behind the incisor teeth and pass it into the stomach without the use of a gag.   The rats are next given the dose of posterior pituitary extract by subcutaneous injection, prepared by dilution of the standard extract 1/100 with saline.   A suitable dose is then 0.2 ml./100 g. of body weight (0.004 unit/100 g.).   The rats are next placed in the cages and the urine collected in 10-ml. graduated cylinders placed below the funnels.   Time is recorded for each group from the mean time of injection of the pituitary preparation, and when the excretion of urine is first observed the amount produced is recorded every 15 min. until it becomes consistently small.   This usually takes about 3 to 3½ hr.

Burn recorded these amounts in tabular form and determined the time

of maximum water excretion by adding together the separate volumes, with the exception of the first collection, dividing by 2 and obtaining the time interval corresponding to this volume from the table. The first collection is rejected because it often occurs as an isolated urine excretion and is not the forerunner of the main diuresis. The test is repeated 2 or 3 days later with the same group of animals in a cross-over arrangement so that those which received the standard will receive the test and vice versa. This is done to compensate for any inherent difference in sensitivity between groups.

The relative potency of the unknown sample is determined from a graph for that particular colony of rats, by previous determinations of the time of diuresis delay with several known doses. Burn's figures are reproduced in Table IV and were used to produce the graph of Fig. 11, taken from his paper and showing the type of curve obtained. The doses on the abscissa are read off to correspond with the mean delay times, for both halves of the cross-over test for the standard and test preparations, and, knowing the potency of the former, that of the latter will be in the ratios of these doses.

TABLE IV

The Antidiuretic Effect of Posterior Pituitary Extract on Fasting Rats—Figures Are the Time in Minutes from the Administration of Water to the Time of Maximum Rate of Excretion

(Burn, 1931)

Dose of pituitary extract injected U./100 g.	Different effects in different groups of four rats	Average effect
0.004	126, 138, 144, 152	140
0.006	140, 145, 178, 163	156.5
0.008	191, 156, 159, 159	166
0.012	210, 174, 163, 175	180.5

Burn emphasized that the calibration curve could not be used for absolute determinations of potency since the general sensitivity of all the animals used at one time could easily differ from that of the ones used for preparing the calibration curve, but the slope of this dose response curve is, in this method, assumed to be constant. Burn gave the figures of 5 comparisons where the true potency was known and showed that the estimated results varied from the true results by up to 13% but had an average variation of 7%. The apparent error was greater when the comparisons were made upon samples of commercial preparations which had been assayed for oxytocic activity, but here allowance must be made for the fact that the oxytocic assays are subject to appreciable errors and also that the antidiuretic activity is closely

correlated with the pressor activity which does not always occur in the same ratio to the oxytocic action.

B. MODIFICATIONS OF THE RAT METHOD

During the last few years many other workers have used and modified Burn's antidiuretic assay. Gilman and Goodman (1937) found that a more consistent response was obtained after the administration of a preliminary hydrating dose of water. This consisted of tap water in a
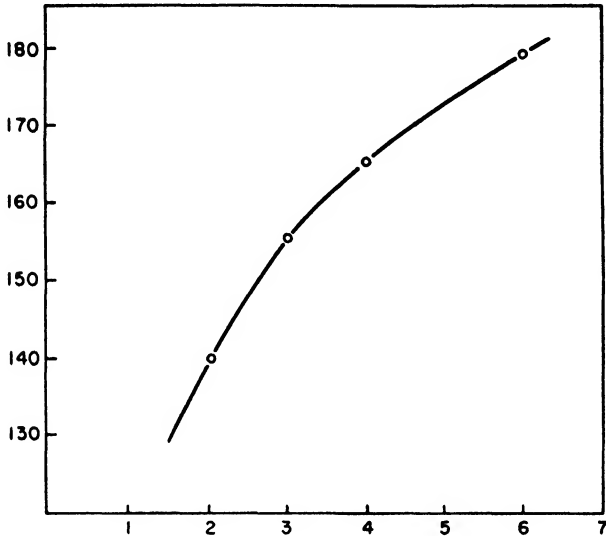
FIG. 11. Dose-response curve obtained by Burn in the assay of posterior pituitary lobe extract using the antidiuretic effect in rats. Prepared from the data in Table IV. The abscissas are arbitrary figures for the dose of pituitary extract. Ordinates, time in minutes from the administration of water to the point of maximum excretion of water. The curve shows the average effect in 64 fasting male rats and may be used to compare the potency of two extracts. (Burn, 1931.)

volume equal to 2.5% of the body weight given by mouth, and, 3 hr. later, at the commencement of the test, all urine previously collected was rejected and twice this dose of water was given at the same time as the dose of posterior pituitary lobe extract. Silvette (1940a) gave a single dose of 10 ml. of 0.2% saline/100 g. by intraperitoneal injection but used a method of calculation based upon the measurement of the total volume of urine excreted per 100 g. of body weight during a period of 6 hr. after the fluid intake. Krieger and Kilvington (1940) used a different method of interpretation again and actually plotted the amounts of urine after intervals of 15 min. against time for a period of 6 hr. from the fluid intake. They then measured the area enclosed by the curve with a planimeter

and used this as the response criterion. They gave less water, using only 12 ml. for a rat weighing 200 g. Robinson and Farr (1940) measured the time taken to excrete 2.5% of the original body weight of water.

It will be seen therefore that, at this time, there was a diversity of slight modifications but there was little evidence as to which was the most accurate. In 1942, Ham and Landis described some research into the antidiuretic effects of urine and pituitrin, and they used a method which was subsequently described by Ham (1943) and was the result of an extensive examination of the methods just mentioned.

Ham used the methods of Gilman and Goodman, Silvette, and Krieger and Kilvington, to produce hydration and the methods of calculation described by Burn, Robinson and Farr, Silvette, and Krieger and Kilvington. Ham also noted that Silvette (1940b) had made the prediction that the effect of the posterior pituitary lobe extracts would be more accurately followed by estimating the excretion of chloride than the antidiuretic action and completed his assays in this way also. He used 18 rats in groups of 3, in cages placed over funnels, the rats being so arranged that there was a difference of not more than ± 10 g. between any group. Of the 6 groups thus formed, 2 were used as controls and 2 for each of the 2 assays. As a result of this comparison, Ham showed that the methods of Gilman and Goodman (1937) and Krieger and Kilvington (1940) were the most reproducible and resulted in the smallest deviations from the true potency. The latter method, however, was then recalculated on a 3-hr. basis, as the 6-hr. time period was unwieldy in practice. Some degree of accuracy was lost but the advantage was considered to be greater. To estimate chlorides Ham used the Volhard method of Van Slyke and Sendroy (Peters and Van Slyke, 1932) and found some quite new phenomena. The water excretion was most satisfactorily measured by the method of Krieger and Kilvington, and in this case the difference in the areas of the curves for the control and the treated rats increased with the increase in the dose of the posterior pituitary extract from 0.1 to 10 mU./100 g. of rat, but above 10 mU. there was less effect due to an increase in water excretion from the brief diuretic action shown by these extracts in the larger doses and not manifest below this level. The chloride excretion, however, increased steadily with increase in dose, and the practical dose range extended from 0.1–100 mU. in this case. The results Ham obtained are reproduced in Table V.

As the result of this work Ham and Landis (1942) used the technic of preliminary hydration with 0.2% saline using a dose of 2.5% of the body weight followed by a second "hydrating" dose of saline (0.2%) equivalent to 5% of the original weight. The pituitary preparations were given by intraperitoneal injection by these workers in a volume

of 1 ml./100 g. of body weight. The urine volume was recorded cumulatively every 15 min. for 3 hr. and plotted as volume/100 g. against time. The area was then measured with a planimeter and became larger after antidiuretic effects were observed so that the difference between the areas for controls and treated rats was usually negative in sign as shown in Table V. Using a scale of 30 min. or 1 ml. equal to 1 in. on all their graphs, Ham and Landis showed that the variation between two control cages on the same day was 1.56 sq. in. on an average value of 11.2 sq. in. for the area measured, using 130 observations to obtain these figures.

TABLE V

The Antidiuretic and Chloruretic Effects of Posterior Pituitary Lobe Extracts upon Rats Previously Fasted and Given Water to Induce Diuresis—the Doses of Pituitary Extract Range from 0.1 to 100 mU./100 g. of Rat

(Ham, 1943)

Pituitrin, mU./ 100 g. of rat	Antidiuretic effect— difference in sq. in. from controls	Total chloride excreted— microequivalents/ 100 g./3 hr.
Saline control[a]	±0.78	Less than 90.0
0.1	+1.2	74.25
0.5	−1.5	109.0
1.0	−3.2	121.0
2.5	−5.3	219.0
5.0	−9.2	252.0
10.0	−10.3	273.0
25.0	−4.9	362.0
50.0.	−5.3	436.0
75.0	−5.0	512.0
100.0	−4.7	653.0

[a] Obtained from 780 observations.

They conclude that the antidiuretic method is satisfactory and accurate from 0.5–10 mU. of pituitrin/100 g., but if the dose exceeds this range and extends to 100 mU. then reliable results are only obtained by estimating the total chloride excretion for the 3-hr. period. This work is a complete study of some of the principal factors involved in the antidiuretic assay method, and it is likely that the only further improvements of importance will be the result of improvements in the statistical design of the experiments and interpretation of the results.

In this connection the split cross-over design (Smith *et al.*, 1944) described more fully in connection with the assay of insulin, would be very suitable. This design enables a determination of the slope of the dose-response curve to be made as an integral part of the determination of potency and will thus counteract alterations in this value from time to time or as the result of using different strains of animals.

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Biological Assay of the Melanophore Expanding Hormone from the Pituitary

By F. W. LANDGREBE and H. WARING

CONTENTS

I. Introduction

The posterior lobe of the pituitary consists of two anatomically distinct structures, *pars intermedia* and *pars nervosa*. The *pars intermedia* manufactures melanophore expanding hormone and the *pars nervosa* a substance (or substances) with oxytocic, pressor, and anti-

diuretic activities.   Unfractionated extracts of posterior lobe exhibit all four activities.

The substance(s) responsible for pressor, oxytocic, and antidiuretic effects has not been isolated in pure form.*   So the activity of an unknown extract is compared with that of an international standard posterior lobe powder (I.S.P.P.) prepared from ox glands according to a specified procedure and kept at National Centres.   Dale (1942) provides a fully documented review of events leading up to the adoption of this international standard.   An international unit of pressor, oxytocic, or antidiuretic activity is defined as that amount of activity present in 0.5 mg. of the I.S.P.P.

We have suggested (Landgrebe and Waring, 1944) that the amount of melanophore expanding activity (henceforth referred to as 'B') in 0.5 mg. I.S.P.P. be also adopted as the international unit, but there has been no formal recognition of this.   In this connection two things are relevant:

1. There is no fixed ratio of melanophore to pressor activity in whole extracts of different ox posterior lobe powders (Table I).

2. The 'B' contents of the first and second international standard powders are almost identical (Tables II and III).

TABLE I

Activity Recorded as Percentage of International Standard Powder
(From Landgrebe and Waring, 1944)

Powder	Pressor	Oxytocic	'B'
1st International Standard	87	87	103
2nd International Standard	100	100	100
Canadian Standard	100[a]	100[a]	40[b]
Substandard used for routine pressor assays	90	80	17
Commercial (Oxo)	65	50	15
Commercial (Duncan Flockhart)	70	70	60
F.G.Y.[c]	60	50	18

[a] Assumed.
[b] Calculated from Stehle's (1936) figures.
[c] A powder kindly supplied by Dr. F. G. Young of the (British) Medical Research Council.

Extracts of posterior lobe pituitary devoid of pressor, antidiuretic, and oxytocic properties have only two certain specific actions (1) chromatophore activation and (2) inhibition of adrenalin hyperglycemia, although several other effects have been attributed to them (Landgrebe and Waring, 1941).   Experiments involving tissue culture of separated

* It is possible that the Van Dyke *et al.* (1942) preparation is pure, but it is not generally available.

intermediate lobe tissue, and experiments with elasmobranch glands in which there is a virtual absence of *nervosa* tissue, have shown directly that the chromatophore activity comes from the intermediate lobe. Evidence that the anti-adrenalin activity comes from the intermediate lobe, and not the nervosa, is still indirect.   Fractions derived from mixed *pars intermedia* and *pars nervosa* tissue which have no specific nervous lobe activities, but are rich in chromatophore excitant properties, contain this property.   Furthermore what evidence we have, although it is not satisfactory, implies that in any extract chromatophore activity is correlated with the anti-adrenalin property.   There is complete evidence for the role of the chromatophore hormone in the color change of intact animals (Waring, 1942; Parker, 1948; Waring and Landgrebe, 1950). Comparable evidence is not available for the antiadrenalin activity, although preliminary evidence implies that this is a physiologically significant agent, and not as we had previously thought, a purely pharmacological one.

Several ways have been described for the biological assay of chromatophore activity, and we are able now to prescribe a routine which will readily give an accuracy of 10%.   Attempts to work out an assay method for anti-adrenalin activity have not been completely successful, and for this reason what follows will be wholly concerned with chromatophore activity.

II. BRIEF HISTORICAL NOTE

A thorough historical account would be inappropriate here but the following selection indicates the major lines of attack on the problem of assaying 'B.'   Abel (1924) ligated posterior limbs of the frog and compared responses after injection of pituitary extract.   McLean (1928) perfused posterior lobe extract through hind legs of the frog and compared the gross color of legs receiving graded doses.   Jores (1933) removed skin from the back of pale frogs and immersed them in different strengths of pituitary extract in Ringer.   Hogben (1924) incidental to his studies on the differential sensitivity of intact and hypophysectomized frogs, used a minimal effective dose method combined with direct reading of the melanophores.

Methods of assay proposed since 1930 have all been based on intact or hypophysectomized amphibians, hypophysectomized *Anolis* or intact *Phoxinus*.   Methods using amphibians have employed a variety of species, the commonest being the frogs *Rana temporaria* (Europe) and *Rana pipiens* (America) and the South African clawed *Xenopus laevis*. Zondek and Krohn (1932) introduced the *Phoxinus* method for assay of their 'B' containing extract which they called Intermedin, because

they found amphibian test animals unreliable. The method using hypophysectomized *Anolis* (Kleinholz and Rahn, 1940) was introduced later than the tests using amphibia, and its authors claimed that it was more sensitive. The relative merits of these different test animals are discussed in the next section.

III. THEORETICAL CONSIDERATIONS

Chromatically active cold blooded vertebrates may have one or all of the following colour cells: dermal and epidermal melanophores, erythrophores and xanthophores containing respectively black, red and yellow pigment. So far as we know all proposed methods of assay have used the melanophores of amphibia or reptiles, with the exception of Zondek and Krohn's method which is based on the activation of erythrophores in *Phoxinus*. Zondek and Krohn proposed their method when they found amphibian material unreliable, but it does not seem to have occurred to them that there might have been different activators (both from the intermediate lobe) for melanophores and erythrophores. Subsequent workers, e.g., see Astwood quoted by Stehle (1938), p. 138, have claimed that there are two excitants, and if this is true then a full assay would demand figures from both methods. Actually, in our hands at least (p. 168) the *Phoxinus* method is too unreliable to permit accurate comparative assays, and it is impossible to decide unequivocally by its use whether one or two hormones are secreted by the intermediate lobe. This question will be considered later.

Specificity is an important consideration in any method of assay. There is good information regarding amphibia, but so far as we are aware, none with regard to other proposed test animals. Hogben's (1924) pioneer work, followed by that of Shen (1937) and Teague *et al.* (1939), has shown that whereas melanophores of the intact frog can be caused to expand by a few non-pituitary substances, a melanophore response in the hypophysectomized frog is specific to pituitary 'B.' Immediately prior to World War II Shen obtained a supply of *Xenopus* from us, and we had a personal note from him saying that it behaved similarly. We concluded from our work on urines, that whereas a few would slightly darken hypophysectomized animals, increased doses would darken them no more. With the one exception noted on p. 167 it is our experience that only 'B' will, in sufficient dose, fully darken a hypophysectomized *Xenopus*.

When a 'B'-containing extract is injected into pale test animals, the melanophores expand and the skin darkens. The skin then gradually gets paler as the melanophores contract. To use this sequence of events for assay purposes we must (1) decide on a method of measuring melano-

phore expansion or darkening of the skin, (2) select a suitable preparation, and (3) adopt a criterion for estimating relative potencies of extracts. These will be considered separately below.

1. Measurement of Melanophore Expansion or Skin Darkening

This can be done by microscopic observation of the melanophores or by naked-eye estimation of the color of the skin as melanophores expand. The objections to the latter, and to Hill's photoelectric method of record-

(a)
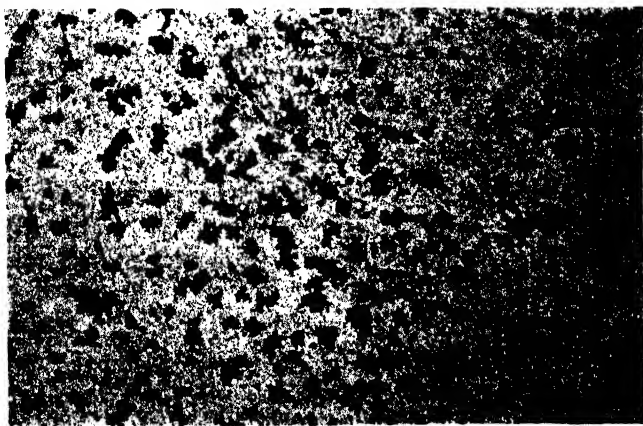
(b)

FIG. 1.   To show how a combination of reduced (1) melanophores per unit area and (2) melanin per melanophore may result in a skin with melanophores equilibrated at melanophore index (m.i.) = 2 being macroscopically darker than skin at m.i. = 5.   Magnification of both photographs the same.   (We have *Xenopus* with less melanin than in (a) but these do not photograph well with the melanophores expanded.) (From Landgrebe and Waring, 1944.)

ing it, have been fully stated (Hogben, 1936, 1942; Neill, 1940). The overriding objection is the variation in melanophore density, which may be so great (as, for example, in the animals used for Fig. 1) that an animal with few but fully expanded melanophores is paler than an animal with abundant contracted ones. Because it relies on gross macroscopic color, the most recent method advocated, viz., *Anolis* (Kleinholz and Rahn, 1940), is suspect. We have no personal experience with *Anolis* because it is not generally available, but since *Anolis* has no webs we see no easy way of circumventing the shortcoming of macroscopic observation. Also it is difficult to imagine that it is as easy to maintain as *Xenopus*.

Quantitative results of real value can only be obtained by a direct estimation of the degree of expansion of melanophores themselves. Hogben, who was the first to realize this, introduced his first melanophore scale in 1922 and an improved one in 1930. Without this the detailed

FIG. 2. Melanophore index (m.i.). (From Landgrebe and Waring, 1944.)

analyses of color change carried out by his school would not have been possible. After a few hours' practice, a novice, with Fig. 2 for reference, can read the melanophore indices (m.i.) of the middle portion of the large web of twelve *Xenopus* without his average differing by more than 0.1 from that of an experienced supervisor. In passing we may note that reading of melanophore state with a very low power microscope as recently suggested has no more to commend it than gross macroscopic reading. A $10 \times$ eyepiece and $\frac{2}{3}$ in. objective form convenient optical equipment.

2. Selection of Suitable Preparations

Three preparations permit easy reading of melanophores: (1) isolated skin immersed in saline, (2) perfused pithed specimens, and (3) intact or hypophysectomized animals with webs that can be quickly positioned on the microscope stage.

(1) and (2) Isolated Skin Immersed in Saline and Perfused Limbs. At first sight the theoretical attraction of both of these preparations is that the principle of internal control could be invoked as in a pressor or oxytocic assay, viz., alternate injection of doses of standard and

unknown. This has so far not proved practicable for two reasons: (a) the effector speed in all investigated cases that have proved suitable on other grounds is so slow that the method would at best be cumbersome; and (b) with *Rana* or *Xenopus* using either perfusions or isolated skin, melanophores, once expanded, do not return to the fully contracted state when saline alone is perfused or the skin is placed in fresh saline; and this aside we have found (Landgrebe *et al.*, in preparation) that there is no satisfactory discrimination between doses. *Scyllium* isolated skin is the only one we have tested in which the melanophores will expand to pituitary extract and then contract again in saline. It will also discriminate between graded doses in a very limited range, but no confidence can be placed in it as test material for assay purposes. Frog skin is unreliable even as a qualitative test because it is sensitive to change of *p*H and other agents (Jores, 1936).

(*3*) *Intact or Hypophysectomized Animals.* Satisfactory assays can be made on *Rana* (and probably other frogs and toads) or *Xenopus*. *Xenopus* is more satisfactory than *Rana* for reasons given below, and all subsequent detailed description relates to this animal. Where only *Rana* is available, the same procedure can be used with obvious minor alterations.

(*a*) *Ease of Reading.* *Xenopus* has a much larger web than *Rana temporaria* and it can more rapidly be positioned on a microscope stage. Chromatophores other than melanophores are either absent or nonobtrusive. The apparent change of shape of the melanophore in transition from 1 to 5 on the melanophore scale is smoother and more uniform throughout the web.

(*b*) *Ease of Maintaining Standardized Conditions.* Temperature, humidity, and light have profound effects on chromatic function of amphibia. All three are easily standardized in an aquatic animal such as *Xenopus*. A serious objection to use of British *Rana* is the dominant role played by humidity: in up to 50% of intact specimens, melanophores are always expanded in presence of water. When the frogs are kept dry, survival is low.

(*c*) *Length of Life.* *Xenopus* lives indefinitely in the laboratory and its maintenance is easy (Landgrebe, 1939). *Rana temporaria* can sometimes be kept alive for a few months, but only with considerable attention. The other advantages of the former are:

1. Under conditions to be specified, their response to a given dose is consistent for long periods (Fig. 3). Thus a rapid rough assay can be made and the appropriate dose chosen.

2. Accurate reading of animals with many melanophores is difficult. This is one of the chief obstacles to accurate assay with the *Rana* avail-

able to us. *Xenopus* kept for long periods on a white background have fewer melanophores (p. 149).

3. Amphibians exposed to conditions that evoke contraction of melanophores (intact animals on a white "background" or completely hypophysectomized animals) gradually lose their pigment (cf. Dawes (1941)), and some melanophores disintegrate. Animals so treated require less 'B' to evoke expansion, i.e., they are more sensitive. Animals that are exposed to conditions that cause prolonged expansion of
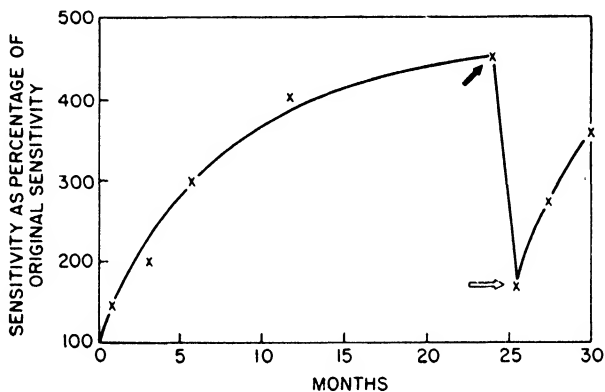
Fig. 3.   Graphic representation of the effect of background on the sensitivity of *Xenopus* to injection of 'B'-containing extracts.   All animals kept on a black background for several weeks before experiment.   For the first 24 months of the experiment toads kept on a white "background."   At → they were transferred to a black background.   At ⇒ they were transferred to a white background.   Points are the average sensitivity figures from same 12 toads.   Sensitivity estimated at intervals shown by injecting each toad equilibrated on a white background with same dose of freshly made extract from the same sample of P.L.P. powder kept in a desiccator at 0°C.   (From Landgrebe and Waring, 1944.)

the melanophores (intact animals on a black "background" or blinded animals in light, or animals with the *pars glandularis* removed) build up pigment, multiply their melanophores, and decrease in sensitivity to 'B' (Fig. 3).   In its incipient stage this loss and gain of sensitivity is not the same for all melanophores in one web.   So that an animal with a varied "background" history spread over a week or so may react to an injection, by some of the melanophores expanding to 2 and others to 5. It is clearly impossible to obtain an accurate assay with such animals. This is prevented by keeping the animals on a white 'background' for a few weeks, after which the melanophores react similarly.   Sensitivity of animals kept on a white 'background' increases fairly rapidly for 12 months.   Then the increase is less rapid and the responses are consistent over a period of weeks.   The increase of sensitivity and decrease of

pigment are both less rapid than the corresponding *decrease* in sensitivity and *increase* of pigment.   Sensitivity and amount of pigment may be correlated so that the increased sensitivity that results from prolonged sojourn on a white background *may* be due to the reduced amount of pigment activated.   We have investigated the change of sensitivity after hypophysectomy and the relevant results are shown in Fig. 4. The sensitivity of operated animals to 'B' injections increases more or less consistently for approximately 6 months, after which the animal is completely reliable.   Comparison of the responses of normal and freshly hypophysectomized animals suggests that the former react to
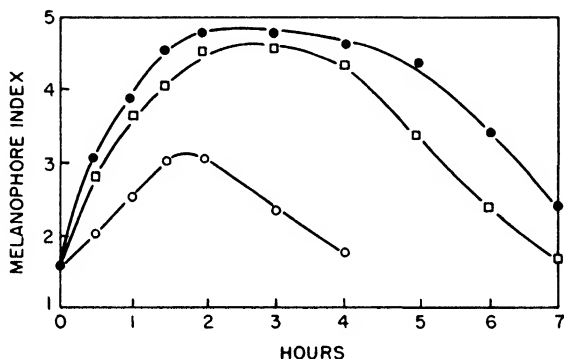
FIG. 4.   Increase of sensitivity after operation of completely hypophysectomized *Xenopus*.   Responses of same group of animals to injection of the same dose of the same extract., 15°C.

—○—   2 weeks after operation.
—□—   2 months after operation.
—●—   4 months after operation.
(From Landgrebe and Waring, 1941)

somewhat smaller doses, but we have not carried out systematic sensitivity experiments on the same animal before and after operation.

Respective merits of normal and hypophysectomized Xenopus for assay purposes.   Assays of all fluids with unknown contaminants, e.g., from blood, urine, should first be done on intact animals which have a far higher resistance to histamine, foreign protein, and other toxic substances.   When dealing with extracts not derived from the pituitary, it is essential to obtain final figures from hypophysectomized animals. Hypophysectomy is simple (Hogben, 1923).

Respective merits of intraperitoneal and dorsal lymph sac injections. We have used dorsal lymph sac (D.L.S.), intraperitoneal (I.P.), subcutaneous and intravenous injections.   Intravenous injections are extremely tedious and quite impracticable for routine work.   *Xenopus*

skin does not readily close over a skin puncture, so subcutaneous injections are unreliable.   Intraperitoneal injections may give erratic results. Sometimes one or two of a group do not respond (probably due to the extract entering the viscera).   If, however, large numbers of test animals are used, fairly consistent results can be obtained.   The response curve after I.P. injection differs from that after D.L.S. injection and varies considerably for different groups of animals (Fig. 5).   Four to ten times the dose is required but greater discrimination is obtained if I.P. injection is used.   However, the results are more erratic and the most satisfactory route for injection is unquestionably the dorsal lymph sac.
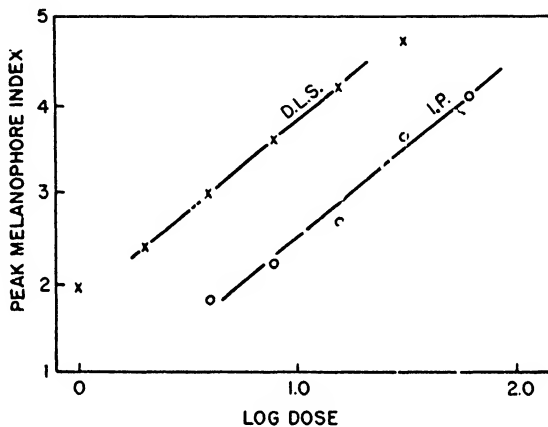
FIG. 5.   Dose response curve of the 18 *Xenopus* used to collect data for Tables II and III.

X—X   Dorsal lymph sac injection.
O—O   Intraperitoneal injection.

Criterion for comparing potency of extracts.   There are two criteria for comparing potency of extract and both have been used (Stehle, 1936; Teague *et al.*, 1939; Teague, 1939; Chen and Geiling, 1943); (1) peak m.i. attained and (2) length of time between injection and resumption of complete pallor.   Standard extracts of dried posterior lobe powders give similar results using either criterion.   We do not use the second for the following reasons:

(*a*) The time response curve is very flat toward the end and there is room for considerable error.   When this method is combined with macroscopic estimation of response, it is not too much to say that the values obtained are worthless.

(*b*) It is not applicable to extracts treated with caustic soda because this treatment flattens the time-response curve (Fig. 6).   The degree

of flattening is not directly proportional to the 'B' content of the original powder (Landgrebe and Waring, 1944; Landgrebe *et al.*, in preparation; Landgrebe *et al.*, 1943).
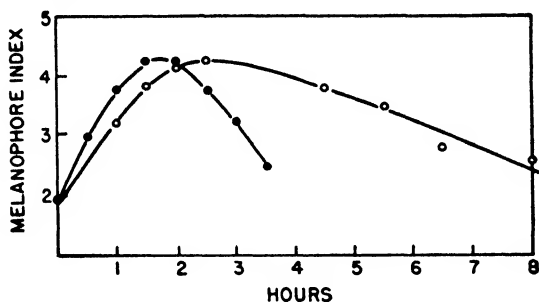
FIG. 6.   Completely hypophysectomized *Xenopus*.   Responses to ox posterior lobe extracts.   All injections 1 ml. 15°C.

—●—   Untreated aqueous extract.
—○—   Same extract treated with caustic soda.   One-half the above dose.
(From Landgrebe and Waring, 1941.)
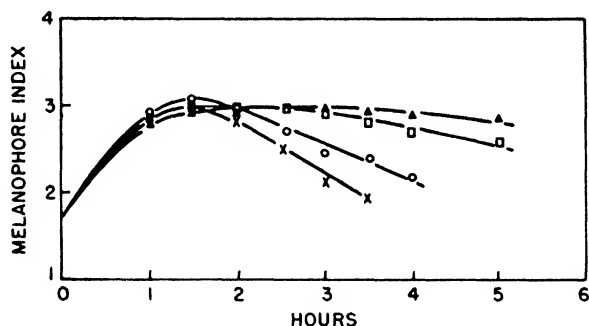
FIG. 7.   Responses of same group of intact *Xenopus* to extracts of whole gland and posterior lobe alone.   Doses adjusted to give approximately same peak m.i. Each point is the average reading from 6 toads.   White background.   15°C.   All doses 0.25 ml./D.L.S.

O = Whole gland extracted by standard procedure with 0.25% acetic acid.
X = Posterior lobe alone extracted by standard procedure with 0.25% acetic acid.
△ = Whole gland extracted by standard procedure with 0.25% acetic acid, neutralized, adjusted to $N/10$ NaOH, heated in boiling-water bath for 10 min. and then neutralized.
□ = posterior lobe alone, ditto.
(From Landgrebe and Waring, 1944.)

(*c*) Inert protein, e.g. presence of anterior lobe tissue, in the extracts flattens the time-response curve even when extraction is made by the standard method with dilute acid (Fig. 7).

(*d*) The second method takes longer than the first.

IV. PRACTICAL DETAILS

1. Selection of Animals

In choosing animals it is important to make certain that they are capable of a maximum response, because some animals exhibit no greater melanophore expansion than is represented by m.i. = 4 − 4.5, even when injected with several times the minimal dose necessary to evoke this index. We have had an occasional animal which, after giving satisfactory service for a period, would temporarily lapse into this type of behavior. The error this can introduce needs no emphasis when looking for peak m.i. attained. In practice, however, for obvious reasons lower dose levels are employed. When dealing with caustic soda-treated extracts (p. 161) inability of melanophores to expand more than to a m.i. = 4 could introduce very big errors in assessing the degree of flattening. A dose, say twice that needed to evoke m.i. = 4, would produce the same rise, but because more excitant would have to be removed from circulation before the index started to decline, a quite spurious flattening of the response curve would appear.

We have performed successful assays on animals weighing from 10 to 200 g., but most workers find toads weighing about 30 g. easiest to handle.

2. Care of Animals

Xenopus should be kept separately in white containers, e.g., 6 in. in diameter, 8 in. high, half-filled with water and fitted with wire-mesh lids. We keep them in a darkened room thermostated at 16°C., with one 40-w. lamp above 24 containers.

At 16°C. *Xenopus* will sometimes eat even when being constantly handled as in assays. Every 6 months or so we rest the animals at 20 to 25°C. when they feed voraciously and rapidly regain any loss of condition (Landgrebe, 1939). Feeding once a week is sufficient, and the containers must be cleaned out next day.

3. Miscellanea with Regard to Assay

i. Dermal melanophores only are used. Animals kept on a white background for 6 months will have a convenient number of dermal and no epidermal melanophores. Otherwise toads with many epidermal melanophores should be discarded as they lead to inaccuracies.

ii. Aim at a dose that will give a peak m.i. around 3.0.

iii. There is a slight gradient of melanophore expansion from the tip of the web proximally, so readings should be taken from the central

portion of the large web to obtain a figure approximating to the average m.i. of the whole web.

iv. Reading is best done with $\frac{2}{3}$-in. objective and 10× eyepiece. Strength of microscope illumination must be adequate and constant.

v. Injections are made with a 2-in. gauge 6 needle, through the thigh muscles. This point of entry is used both for D.L.S. and I.P. injections (Fig. 8).
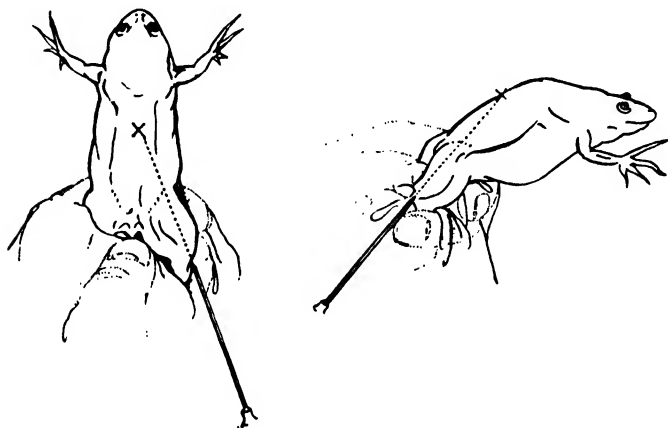
FIG. 8. Injection into the dorsal lymph sac. The animal is held with a cloth as shown. The point of the needle, bevel uppermost, is passed through the muscle, tilted upwards, and directed to the point × just under the skin of the back. (From Landgrebe, 1949.)

vi. As many as 20 D.L.S. injections can be made with water as a solvent. More than this often results in a large dark area appearing on the back ("jacketing") and the melanophore index in the web does not rise. This is because the injected activity does not leave the lymph sac owing to inactivation of the lymph heart. Jacketing can be avoided by using the following saline as a solvent:

	Total Content			*Prepare as follows*	
NaCl	8.3 g.		Soln. 1.	NaCl	8.5 g.
KCl	0.33 g.			KCl	0.34 g.
CaCl$_2$	0.12 g.			H$_2$O	1000 ml.
NaHCO$_3$	0.1 g.		Soln. 2.	CaCl$_2$6H$_2$O	0.24 g.
MgCl$_2$	0.1 g.			H$_2$O	10 ml.
Dextrose	0.5 g.		Soln. 3.	MgCL$_2$6H$_2$O	0.22 g.
H$_2$O	1000 ml.			H$_2$O	10 ml.

Just before use mix 100 ml. of Soln. 1, 1 ml. of Soln. 2, 1 ml. of Soln. 3, and add 0.01 g. NaHCO$_3$, and 0.05 g. dextrose. Animals that jacket

in spite of the use of this saline have damaged lymph hearts and must be given I.P. injections.

vii. Claims have been made that the volume of fluid used as a solvent for injected activity affects the response (Callaway *et al.*, 1942). We cannot confirm this. Nevertheless it is advisable to standardize the volume injected. We find that 1 ml. and 0.25 ml. give identical responses and suggest the use of 0.25 ml.

V. Performance of an Assay

1. Preliminaries

i. Select a large sample of posterior lobe powder preferably prepared from fresh glands and stored according to standard procedure (Burn, 1937). Use this as substandard. There is no reason to suppose thât such a preparation deteriorates even over a period of 13 years (cf. 1st and 2nd International Standard Powders, Dale (1942), and Table III of this paper).

ii. Prepare a standard extract of substandard powder with dilute acetic acid (British Pharmacopoeia, 1932, 1948). Such extracts unsealed are stable for a few hours and then sometimes deteriorate rapidly. Stehle (1936) also noted this instability of *some* extracts. Solutions store well if ampouled and placed in a B.W.B., sealed, and kept in the dark. At room temperature they are stable for at least 3 months and at 0°C. for at least 6 months.

Alkali-treated extracts must be standardized against extracts of international standard powder subjected to similar treatment at the same pH (p. 164).

iii. Divide 12–24 toads into 2 equal groups, *A* and *B*.

2. Standardization of Substandard Powder

Inject suitable doses (refer for guidance to Tables II and III) of the standard into test animals of group *A* and substandard into those of group *B*. On the next day inject the same dose of standard into *B* and of substandard into *A*. Repeat this procedure at three dose levels. This will yield data that can be used for (1) constructing a skeleton dose-response curve and (2) calculating the potency of substandard to about 10% (cf. Tables II and III). To construct an accurate dose-response curve several dose levels must be used. Figure 9 shows the kind of discrimination to expect between different doses.

If a large number of routine assays are not contemplated and a figure correct to about 20% is sufficient, a simpler procedure can be adopted. Inject group *A* with standard and group *B* with the unknown

TABLE II

Assay to Determine the Number of L.W. Units per Milligram of International Standard Powder

1. Two groups (C and D) of 6 intact Xenopus selected for approximately equal sensitivity and from the same group used to construct the dose-response curve (Fig. 5).

2. Initial extractions of powder with dilute acetic acid as prescribed on p. 8 of *Med. Res. Council Bull.* (1936) and British Pharmacopoeia. Dilutions made with saline prescribed on p. 153.

3. All injections 0.25 ml. D.L.S. All assays on white background with constant overhead illumination 15°C.

(From Landgrebe and Waring, 1944)

Date	First international standard powder					L.W. standard powder					
	Group of 6 toads	Dose in µg.	Max. m.i.	Average max. m.i. from 12 toads	Relative dose read from Fig. 5	Group of 6 toads	Dose in L.W. units	Max. m.i.	Average max. m.i. from 12 toads	Relative dose read from Fig. 5	L.W. units/mg. International Standard Powder
27.5.43	C	1.25	3.5	3.25	1.3	D	1.25	3.0	3.1	1.15	$\frac{1.3}{1.15} \times \frac{1000}{1} = 1130$
28.5.43	D	1.25	3.0			C	1.25	3.3			
29.5.43	C	0.625	2.7	2.5	0.55	D	0.625	2.4	2.6	0.6	$\frac{0.55}{0.6} \times \frac{1000}{1} = 916$
30.5.43	D	0.625	2.3			C	0.625	2.8			
26.6.43	C	1.25	3.3	3.25	1.3	D	1.25	3.2	3.3	1.4	$\frac{1.3}{1.4} \times \frac{1000}{1} = 930$
27.6.43	D	1.25	3.2			C	1.25	3.4			

TABLE III

Assay to Determine the Relative 'B' Content of the First and Second International Standard Powders
Two groups (A and B) of 12 intact Xenopus from the same group as C and D of Table II. All conditions same as in Table II.
(From Landgrebe and Waring, 1944)

	First international standard powder					Second international standard powder					Second international standard as percentage of first international standard
Date	Group of 12 toads	Dose in milli-units	Max. m.i.	Average max. m.i. from 24 toads	Relative dose read from Fig. 5	Group of 12 toads	Dose in milli-units	Max. m.i.	Average max. m.i. from 24 toads	Relative dose read from Fig. 5	
18.5.43	A	5.0	4.1	4.05	3.5	B	5.0	4.0	4.0	3.2	$\frac{3.2}{3.5} \times 100 = 91$
19.5.43	B	5.0	4.0			A	5.0	4.0			
20.5.43	A	2.5	3.2	3.2	1.25	B	2.5	3.2	3.15	1.2	$\frac{1.2}{1.25} \times 100 = 96$
21.5.43	B	2.5	3.2			A	2.5	3.1			
22.5.43	A	1.25	2.4	2.4	0.5	B	1.25	2.6	2.45	0.53	$\frac{0.53}{0.5} \times 100 = 106$
23.5.43	B	1.25	2.4			A	1.25	2.3			

extract at one dose level (preferable a dose that raises the m.i. to about 3). Calculate the result using Fig. 5 of this paper. Experience shows that the sensitivity of toads varies considerably, but the log dose-response curve of any group for *dorsal lymph sac* injections is not significantly different from that illustrated. This is strikingly demonstrated by comparison of graph 6 in Waring and Landgrebe (1941), which is based on observation of hypophysectomized toads by Waring in 1940, and
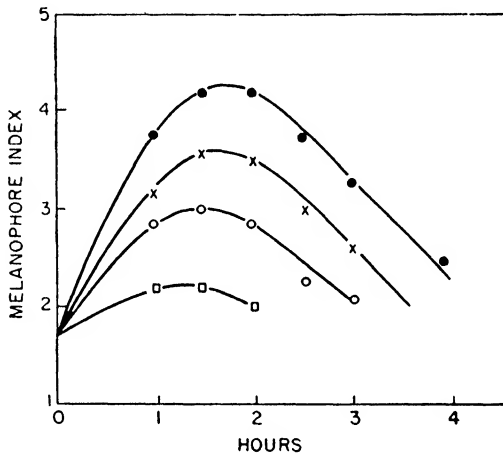
Fig. 9.   To illustrate the response of intact animals to graded doses of the same 'B'-containing extract (supplied by Prof. Stehle).   15°C.   White background.   All injections ¼ ml. D.L.S.

● = 0.2 μg.,   X = 0.1 μg.,   O = 0.05 μg.,   □ = 0.025 μg. Stehle's powder.
(From Landgrebe and Waring, 1944.)

which conforms to Fig. 5 shown here, based on normal animals read by Landgrebe in 1942.

3. Assay of an Unknown Extract

It must again be emphasized that hypophysectomized animals must be used for other than pituitary extracts.

Prepare 3 or 4 trial doses of the unknown and inject each into one toad.   At 16°C. read at 1½ hr. (peak rise).   From the figures so obtained estimate a dose that will raise the average m.i. of a group to about 3.0. Avoid doses that raise the index above 4 because sometimes melanophores temporarily lose their ability to attain 5.0.   Inject the unknown extract into group *A* and a comparable dose of substandard into group *B*. Reverse the groups next day.   The response to substandard shows whether the toads are behaving true to form.   If they are, the potency of

the unknown extract can be read off on the dose-response curve (cf. Tables II and III).

VI. Sample Assays

1. In our earlier work we used a specially prepared powder as a standard, and an L.W. unit was an arbitrary amount of this. Table II contains data from an assay to determine the number of L.W. units per milligram of international standard powder. It shows that 1 mg. of international standard powder contains about 1000 L.W. units, i.e., 1 L.W. unit = 2 "international" units.

2. Table III shows a comparison of the first and second international standard powders. The second, which is now the standard, has about 97% of the activity of the first.

VII. Accuracy to be Expected

There are two main sources of error in assay methods of this kind: (1) mistakes in reading and (2) inconsistency of response over the period (2 or 3 days) necessary to a complete assay. The first has been mentioned on p. 146, and the error from this source has been estimated in the following experiment:

A worker was trained to read melanophore indices to 0.5 on the scale, so that an index between, say, 3 and 4 was recorded as 3.5. A group of ten toads which had been equilibrated on a black background, were then transferred to a white background and the melanophores of each toad was read 12 hr. later. The melanophores were then re-examined and five successive records taken for each toad. In the period of time necessary for these observations the melanophores of the animals contract very little on the white background, as the natural color change of the animal at this stage is fairly slow. The grand mean for the 50 readings was 2.72, and the maximum probable error calculated for the group means was only 0.08. This error taken in conjunction with the dose-response curve (Fig. 5) would produce a maximum final error of only about 10% for a reading of 10 toads. So that in a usual assay using 24 animals the error from this source is much less than 10%.

The second source of error, consistency of response, can best be illustrated by three examples:

(a) *Over short periods.* The consistency of response by *selected* toads over short periods of time is illustrated by groups *A* and *B* (Table III) at three dose levels spread over 6 days.

(b) *Over long periods.* See Fig. 3.

(c) *With toads taken at random.* Two groups of 6 were taken at random from a tank of 30 used for pregnancy diagnosis. Each animal

received 0.25 ml. of a 1 in 200 dilution of standard extract of I.S.P.P. into the D.L.S. with the following result:

Time	0	1 hr.	1½ hr.	2 hr.	3 hr.
Group 1 (average m.i.)	1.7	2.7	3.1	3.2	2.3
Group 2 (average m.i.)	1.5	2.9	3.5	3.5	2.8

A smooth curve drawn through these points shows the maxima to be 3.2 and 3.5 respectively. Taking these as the criteria of potency and assuming the dose-response curve in Fig. 5, the ratio of the doses recorded from this test is 1:1.3.

The same dose was administered the next day with the following result:

Time	0	1 hr.	1½ hr.	2 hr.	3 hr.
Group 1 (average m.i.)	1.5	3.0	3.3	3.2	2.4
Group 2 (average m.i.)	1.4	3.0	3.6	3.4	2.7

The maxima, 3.3 and 3.6, are almost the same as those of the previous day. The maximum of group 1 first day averaged with that of group 2 second day is 3.4. The maximum of group 2 first day averaged with that of group 1 second day is 3.45. On the dose-response curve these figures give a difference between the equal doses of about 10%.

The data show remarkable consistency of response, but it must be emphasized that the background history of all these toads had been the same for at least a year, so one of the greatest potential sources of inaccuracy was eliminated.

VIII. IMPLICATIONS OF DISPROPORTIONATE 'B'/PRESSOR AND 'B'/OXYTOCIC RATIOS

Data in Table I demand further examination on two issues.

1. The Low 'B'/Pressor Ratio of Most Powders

There is convincing evidence that 'B' emanates from the *pars intermedia* and pressor, oxytocic, and antidiuretic activities from the *pars nervosa*. There is yet no evidence that ox pituitary powders *prepared according to standard procedure* have a pressor/oxytocic ratio different from that of I.S.P.P. Nor is there any indication that a relaxation of "standard" conditions (e.g. delay between killing and immersion of gland in acetone, etc.) results in differential loss of oxytocic and pressor activities. Table I shows that the 'B' pressor ratio, however, is not constant in various samples of powder. There may be two reasons for this:

1. Since 'B' and pressor come from different parts of the gland it may be that the size ratio of these two parts varies. So the discrepancy

noted may be due to different quantities of 'B' and pressor in the original gland. There is no evidence for this.

2. That with these other powders some relaxation of the prescribed methods of preparation or storage may have resulted in greater loss of 'B' than of pressor activity. There is no direct evidence for this either, but two reasons predispose us to consider it more likely than (1) above. They are: (a) close similarity of the 'B' pressor ratio of the two international powders prepared in different countries at a thirteen years' interval; (b) in all powders, except apparently the Canadian S.P. where the 'B' potency is low, the pressor figure is also below standard. Of the several ways by which relaxation of the standard procedure might lead to greater loss of 'B' than of pressor activity, we cannot explain the low 'B' content of two examples by diffusion of activity into the anterior lobe (Table IV).

TABLE IV

Activity Expressed in "International" Milliunits of 'B' per Milligram of Powder
(From Landgrebe and Waring, 1944)

	A.L.P.	P.L.P.	Whole gland[a]
Commercial (Oxo)	100	300	130
F.G.Y.	60	320	100
International preparation A.L.P.[b]	50	....	...
" " P.L.P.[b]	...	2000	375

[a] Assuming the A.L.P. is five times the weight of its P.L.P.
[b] Although both these powders were produced by Armour, we have no information as to whether they were prepared from the same glands.

2. The Effect of Various Activities on Each Other with Reference to the Accuracy of Assays

The possibility that the ratio of pressor, oxytocic, and antidiuretic activities in substandard powders may be different is envisaged in the Memorandum of the National Institute for Medical Research (1936/43). This memorandum does not discuss the possibility that a disproportionate amount of one of the three activities might affect the assay of another. The relevant literature is meager.

There is some evidence that oxytocin inhibits the coronary constriction caused by large doses of the pressor activity, but no information is available to show that the pressor assay is significantly affected by a disproportionate ratio of pressor and oxytocin in the doses usually injected. Kamm *et al.* (1928) found that their pressor fraction evoked the same blood pressure rise in the anesthetized dog when injected alone or mixed with an equal number of oxytocic units. Smith (1943), using various mixtures of pressor and oxytocin, showed that the "pressor

principle in preponderant amounts does not inhibit the action of the oxytocic on the isolated uterus."

There is no experimental evidence to show that the presence of 'B' affects assays of pressor, oxytocic, or antidiuretic activities.   Earlier work (Landgrebe and Waring, 1941) showed that the presence of pressor activity in doses of a 'B' extract reduces the time taken for the melanophores to contract once the peak response is passed.   To determine whether pressor activity reduced the *peak response* to 'B' it is necessary to test the effect of pressor-free 'B' before and after the addition of known amounts of vasopressin.   Previously this was not possible because we had no pressor extract substantially free from 'B.'   We now have an extract that contains less than 1 I.U. of 'B,' 20 I.U. pressor activity, and 100 I.U. of oxytocin.   The amount of 'B' in this extract was assayed after treating it with caustic soda.   Over 95% of the pressor and oxytocic activity was thus destroyed and the amount of 'B' present after treatment was determined without the possible interfering effect of large doses of vasopressin and oxytocin.   Experiments with this extract show that within the range of mixtures of neural lobe activities and 'B' likely to be encountered, the former have no effect on the latter *when peak m.i. is the criterion of potency.*

We may conclude, therefore, that it is legitimate, if expedient, to keep one substandard of posterior pituitary in the laboratory for the assay of both neural lobe activities and 'B,' provided it is assigned a separate figure of potency relative to that in I.S.P.P. for each activity (cf. Medical Research Council (British), 1936/43, p. 9).

IX. Assay of Material That Has Been Subjected to Caustic Soda Treatment

Considerable work has been done with glandular extracts subjected to caustic soda treatment because this destroys the pressor and oxytocic properties and increases 'B' potency.   When unfractioned extracts of ox posterior lobe are heated in a boiling-water bath for 2 min. at pH 13.0, two things happen: (1) a greatly increased melanophore expanding potency as judged by peak m.i. attained, and (2) a pronounced increase in duration of response when judged on sub-maximal responses (Fig. 6). These two effects have been analyzed elsewhere (Landgrebe *et al.*, in preparation).

For our present purpose we may note that (1) has no relation to the destruction of the pressor component which accompanies it, and with different species of donor the effect may be very different in magnitude; to save periphrasis we have designated this effect "potentiation."   The increased duration of response (2) does *not* parallel potentiation so the

two effects are different; duration of response is increased more in extracts containing much inert material though the effect is not chiefly attributable to this (Table V and Fig. 7). We have called this effect "protection" because indirect evidence implies that it results from reduced rate of destruction in the body.

The exact mechanism of potentiation and protection are not understood but the following observations are relevant here.
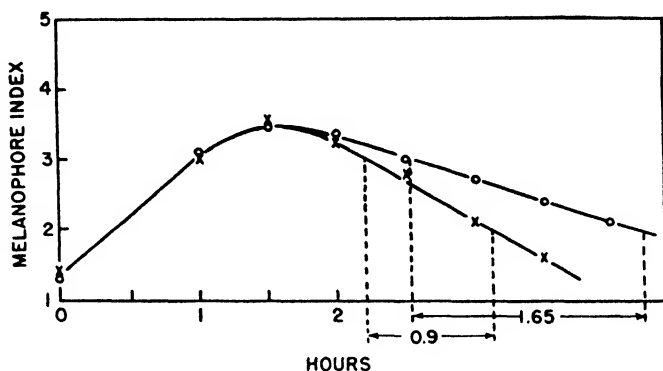
FIG. 10. $\times$ = average m.i. 12 *Xenopus* injected with I.S.S.E. untreated—diluted 1 in 120 and 0.2 ml. injected.

$\bigcirc$ = average m.i. same 12 *Xenopus* injected with I.S.S.E. treated—diluted 1 in 240 and 0.2 ml. injected.

Treatment = adjustment to pH 13 and BWB for 2 min.

Since same peak m.i. is attained after injection of half the dose of treated extract, *potentiation* = *100%*.

Since time taken for m.i. to fall from m.i. 3 to 2 is 0.9 hr. after untreated extract and 1.65 hr. after treated, protection $= \dfrac{1.65 - 0.9}{0.9} \times 100 = 83\%$. (From Landgrebe *et al.*, 1950.)

1. Potentiation. Since 'B' activity after charcoal treatment no longer potentiates when treated with caustic soda, we have assumed that the full effect of a potentiated extract is due to 'B' + something else we have called B_1. The optimum treatment for formation and destruction of B_1 has not been worked out. 'B' after adsorption to carbon, and so freed from the precursor of B_1, is destroyed by caustic soda treatment, so it seems likely that when simple 'B'-containing extracts are potentiated by caustic treatment both destruction and potentiation are progressing concurrently. The relevance of all this to assay methods is that with so many conflicting agencies at work, no comparison of two extracts, e.g. standard and unknown, is valid unless both have had *precisely* the same treatment. Lastly, with regard to potentiated extracts we must note that whereas *intact animals with a short history on a white background*

measure B_1, *those maintained in a pale state for years do not.* We do not know (*a*) whether this effect is graded or "all or nothing," (*b*) whether animals hypophysectomized for long periods lose their ability to respond to B_1.

We have suggested that in a full assay the untreated extract should also be compared with a caustic treated, i.e., potentiated, one from the same original material and their relationship expressed as shown in Fig. 10 (cf. Table V).

TABLE V

Effect of Caustic Soda Treatment on a Selection of Original Glandular Materials

1 ml. of 0.25 % acetic acid extract + 0.14 ml. N NaOH, B.W.B. 2 min., 0.1 ml. NHCl (neutralizes to litmus).

Original extracts approximately equivalent in 'B' activity to that of a standard extract of I.S.P.

(From Landgrebe *et al.*, 1950)

Extract	Percentage potentiation	Percentage protection
Ox I.S.P. (P.L.P.)	110	80
Ox commercial (P.L.P.)	100	80
Ox commercial (A.L.P.)	20	160
Dogfish (P.L.P.)	220	80
L.R.W.₂ (Landgrebe *et al.*, 1943)	0	0

2. Protection. Caustic soda treatment of 'B' after its adsorption to, and elution from charcoal does not produce a "protected" extract (Landgrebe and Waring, 1941), but treatment of all other B-containing extracts does so in varying degree. *All test animals, whatever their background history, exhibit protection.* We have suggested a method for expressing the amount of protection obtained (Fig. 10).

In view of the information set out in this section we need not bother to criticize in detail schemes to measure potency that involve caustic soda treatment of extracts (Chen and Geiling, 1943), particularly when estimates of alleged potency are made by measuring duration of response. Furthermore, although gross macroscopic color determination is to be deprecated in assay work at any time, it is here seen at its worst because (*a*) the end point is so prolonged by this method, and (*b*) it is quite impossible to be certain that one is dealing with a submaximal response (cf. p. 152). In any case most workers who have employed caustic treatment alone without parallel assays of untreated material have heated for varying periods up to 10 min. after adding variable amounts of caustic soda. The pH and time of heating are critical for optimum results (Tables VI and VII). No potentiation or protection occurs if the extracts are heated at pH's below 12, and although we have no exact data about what happens at higher pH's it seems likely from information

given in Table VII that destruction of activity would mask any potentiation. In the absence of evidence to the contrary, it seems likely that from the start of heating with caustic soda potentiation and destruction are proceeding simultaneously, and until a full analysis is available, the only safe procedure in assay is to treat standard and unknown in precisely the same way and also to record *precisely* the procedure followed.

TABLE VI

Potentiation and Protection at Different pHs

Standard extract International Standard Powder 0.1 ml. + 0.9 ml. buffer (glycine) adjusted to *p*H as measured (with cation correction) on Marconi glass electrode meter and 2 min. in B.W.B.

(From Landgrebe *et al.*, 1950)

pH	Percentage potentiation	Percentage protection
8.0–12.0	0	0
12.5	30	50
12.6	50	50
12.8	100	70
13.0	100	80

TABLE VII

Effect of Heating a Standard Extract of International Standard Powder for Different Times at pH 13 (pH Adjusted with N NaOH and Measured with a Glass Electrode)

(From Landgrebe *et al.*, 1950)

Duration treatment in minutes	Percentage oxytocic activity remaining	Percentage potentiation	Percentage protection
1	30	100	80
2	5	100	80
3	1	80	80
7	0	50	75
10	0	−20[a]	60

[a] i.e., slight loss of potency.

X. ASSAY OF 'B' IN URINE, BLOOD, AND TISSUE EXTRACTS

It is convenient to discuss this under two heads: (1) cold-blooded vertebrates that exhibit color change and (2) warm-blooded animals.

1. Cold-Blooded Animals

The procedure for estimating the blood concentration of intact uninjected animals is simple. The blood is merely drawn, agitated to prevent clotting or serum taken from it, and injected intraperitoneally into a hypophysectomized animal of the same species as the donor. A sample preliminary graph is shown (Fig. 11). In this way difficulties with protein shock are avoided and it is possible to estimate the amount of 'B' circulating in intact animals under various conditions. So far

as we know no estimates have been made of the 'B' content of tissues, but there is no reason to believe that simple aqueous or acid extracts would not be effective for this.

We have made assays on urine from untreated *Bufo*, but no activity could be detected in the urine from either normal animals or from toads previously given by intraperitoneal injection a dose of 'B' sufficient to darken 1000 *Xenopus*.
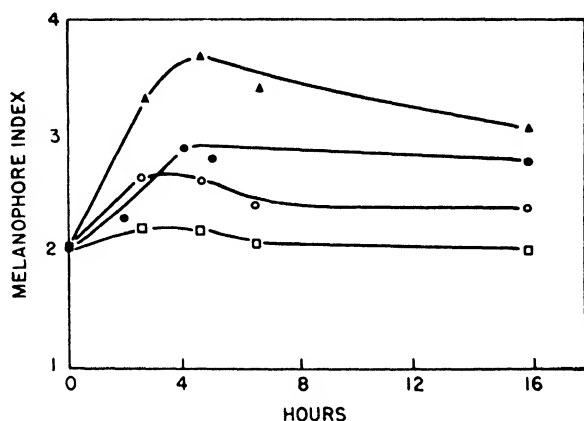
FIG. 11.   Responses of hypophysectomized eels to doses of pressor-free posterior lobe extract and serum removed from black adapted eels.   All injections 3 ml.

—●—●—   Serum from donor fish.
—△—△—   0.01 % pituitary extract.
—○—○—   0.005% pituitary extract.
—□—□—   0.0025% pituitary extract.
(From Waring and Landgrebe, 1941.)

2. Warm-Blooded Animals

The difficulty here is that no warm-blooded animal has been described with functional melanophores, so that a straight transfer from a donor to a test animal of the same species is useless.   Blood transferred direct from rabbits, etc., to *Xenopus* is toxic.   We have tried several procedures such as heating serum, boiling with acid, etc., but the one we find to give the highest yield as judged on blood samples to which have been added known quantities of 'B' is as follows.

Blood is run into a large volume of acetone and the precipitate so formed is dried and stored over P_2O_5.   The dried powder is ground and extracted with boiling absolute alcohol which is then evaporated to dryness and the residue taken up with water and injected.   In experiments involving additions of known amounts of 'B' to the blood before extraction the yield is about 60%.   Since loss is bound to occur at least

three stages in processing, this low yield is not surprising. If water or 70% alcohol is substituted for the absolute alcohol in an effort to gain a higher yield, a toxic extract results. Re-extraction of the residue by boiling with 0.25% acetic acid, and attempts to recover 'B' from the acetone yielded little activity. Another method was also used with similar results. Blood was run into 4 volumes of absolute ethyl alcohol and heated for a few minutes in a boiling-water bath. The extract was centrifuged while hot, the supernatant fluid poured off and evaporated to dryness in a boiling-water bath. The residue was taken up in a few milliliters of distilled water and injected immediately.

In most experiments with normal donor animals we used 3 ml. of blood, but in some cases up to 6 ml. was drawn. In no case did we get a melanophore response which could undoubtedly be ascribed to 'B.' This has not been the experience of other workers in this field. Jores (1935) reported an increase of melanophore excitant in the blood of rabbits kept in darkness. Levinson (1940) detected a pronounced diurnal fluctuation in the melanophore excitant content of rat blood. Jores (1935) did not detail his technic, but in a previous paper (Jores, 1933), he described the precipitation of blood by acetone, extraction with dilute acetic acid, evaporation to dryness, solution of the active principle in hot absolute alcohol, evaporation to dryness, solution of the residue in water and assay on isolated frog skin. The differences he noted were found whether the blood extract was subjected to caustic soda treatment or not. We have previously expressed doubt as to the validity of assays based on isolated skin. Further doubt is cast on Jores' work by the fact that his data imply that he was dealing with small differences based on degrees of melanophore expansion which might be easily produced by substances other than 'B.'

Levinson's data are of particular interest because he used very small quantities of blood (0.3 ml.). His technic, however, is open to serious criticism. He treated all his blood extracts with caustic soda and determined the relative potency of various extracts by the length of time pale frogs remained dark after injection. The objections to the use of caustic soda in this way have been stated already.

In our experiments a very noticeable local darkening frequently occurred and persisted for various periods, even though the melanophore index did not rise above 2.5. The reason why we are doubtful as to whether we ever detected 'B' in the blood of normal animals is that if the equivalent of 3 ml. raised the index to 2.5, twice the dose had no greater effect. This does not happen with graded doses of pituitary extract. Urine from mice, rabbits, and men is well tolerated by the test animals, but results are negative both with urine from normal

animals and from those given large intramuscular or intraperitoneal injections of 'B' extract. After intravenous injection of large doses, however, 'B' could be readily detected in the urine.

During work with urines we came across one sample which contained a substance which could activate the melanophores of a hypophysectomized toad and which we think was not of pituitary origin. Large quantities of this urine were treated with charcoal and after filtration the urine was still as potent as previously. We were not able to extract any active principle from the charcoal even though the phenol elution was prolonged for 24 hr. at 50°C. As a test of method for small quantities of hormone, 50 μg. of a purified powder was added to 200 ml. of the neutralized urine. Over 30 μg. was recovered when phenol elution was prolonged for 24 hr. at 50°C.

In the course of work on the metabolism of the hormone we estimated that 'B' content of liver tissue. Liver was removed from untreated rabbits and guinea pigs, macerated, and boiled in water. Unboiled liver extract had no effect. The boiled and filtered extract was well tolerated by intact and hypophysectomized *Xenopus* and the melanophore index of both was raised to 5 by a sufficiently large dose. On cooling a boiled extract the activity is readsorbed to some extent. It was found possible to adsorb this excitant to charcoal. These two criteria, ability to raise the melanophore index of hypophysectomized *Xenopus* to 5 and adsorption and elution from charcoal are as far as we can go at present in identifying the excitant substances from liver and pituitary as the same.

XI. The Question of Whether the Pars Intermedia Manufactures Two Separate Chromatophore Excitants

In 1932 Zondek and Krohn introduced the *Phoxinus* test for the chromatophoric pituitary excitant claiming that it was preferable to the frog test because it was specific and the *p*H of the injected fluid did not influence the response evoked. None of their explicit and implied claims has been substantiated. The specificity of amphibian response is high (pp. 144, 168), it is certainly not influenced by small changes in the *p*H of the injected fluid, and the specificity of *Phoxinus* response is not good (Collin and Drouet, 1934; Stutinsky, 1934). So the chief interest in Zondek's test is that it may be a measure of an intermediate lobe activity which is different from the evoking melanophore expansion in Amphibia. In their original publication Zondek and Krohn did not suggest that erythrophores and melanophores were activated by two separate substances. There is, however, a body of observation which although open

to criticism on several counts implies that further investigation would be justified. These observations are:

1. Activation of erythrophore excitant is brought about by acid; activation of melanophore hormone by alkali; boiling with alkali increases melanophore potency and decreases erythrophore potency.

2. Erythrophore hormone but not melanophore hormone is readily soluble in absolute ethanol and boiling sodium chloride solution extracts erythrophore hormone in the residue.

3. Extracts from different parts of ox gland contained different proportions of the two properties; *pars intermedia* is richest in erythrophore expanding substance; basophil area richest in melanophore hormone (Jores and Lenssen, 1933; Jores and Will, 1934).

4. Extract of pituitaries from frogs maintained in complete darkness evoked little response when injected into frogs but caused erythrophore expansion in *Phoxinus* (Rodewald, 1935).

5. Astwood observed that a melanophore-exciting preparation similar to Stehle's when treated with alkali had enhanced melanophore excitant properties and reduced erythrophore activity (Stehle, 1938).

With regard to these data we make these comments:

1. All Jores' *quantitative* work is suspect because he used an unsatisfactory assay method (isolated skin).

2. Rodewald's observation is explicable on threshold grounds, but in any case we have shown repeatedly that a frog pituitary under the conditions cited is rich in melanophore excitant activity.

3. We do not know how Astwood assayed his melanophore excitant activity, but he used Zondek and Krohn's unsatisfactory method for estimating erythrophore exciting activity.

Despite these adverse criticisms there is, as we said previously a *prima facie* case for re-investigation. So we injected *Phoxinus* with extracts not subjected to caustic treatment (Stehle) and one subjected to caustic soda (LRW₁), and the results are shown in Table VIII. The animals that did not respond to 0.001 mg. of Stehle's preparation were unresponsive to 5 times the dose. Clearly, as performed by us, the test is valueless, and the only conclusion that can be drawn from it is that caustic soda-treated extracts can activate erythrophores.

Further work on this problem will require modification of the *Phoxinus* test along at least three lines: (1) a *Phoxinus* unit was defined as the amount which would cause red patches on 4–9 sq. mm. to appear between the pectoral and pelvic fins and around the anal fins; this will need to be brought into line with modern practice so that a unit is defined in terms of I.S.P.P.; (2) if, as seems probable, the response is conditioned by the sex phase of the animal this will have to be controlled; (3) rejection of

macroscopic color matching in favor of direct estimation of chromato-
phores as indicated by Bottger (1936, 1937).

TABLE VIII

An Assay Using Phoxinus.   (*The Same 12 Phoxinus Were Used for All Extracts*)
(From Landgrebe and Waring, 1944)

Dose	"International" milli-units of 'B'	No. *Phoxinus* injected	No. showing ventral red patches
Stehle preparation			
(a) 0.001 mg./fish	20	12	8
(b) 0.005 mg./fish	100	12	7
L.R.W.₁ preparation			
(a) 0.00012 mg./fish	20	12	8
(b) 0.0006 mg./fish	100	12	8

XII. Summary

1. A brief survey is given of methods that have been proposed for the
assay of pituitary melanophore expanding ('B' or intermediate lobe)
hormone.

2. Detailed instructions are given for assay of 'B' hormone in glandu-
lar extracts and body fluids using *Xenopus laevis* as a test animal.   Ten
per cent accuracy is quickly and easily obtained.

3. The criterion of potency is the maximum melanophore index
attained after injection of extracts into the dorsal lymph sacs of fully
pale intact or hypophysectomized animals.

4. Intact *Xenopus* kept on a white background for long periods sus-
tain an actual loss of pigment and their sensitivity to pituitary extracts is
increased.

5. The second international standard posterior lobe powder contains
about 97 per cent of the melanophore-expanding activity of the old
standard.

6. We suggest that an international unit of melanophore activity be
defined as that amount in 0.5 mg. of the international standard powder.

7. The ratio of melanophore-expanding, pressor, and oxytocic activi-
ties is not the same in all ox posterior lobe powders.

8. The maximum melanophore index evoked by injection of a pos-
terior lobe extract is not influenced by the amounts of pressor and
oxytocic activity normally present.   So one substandard powder may
be used for assay of all activities, provided a separate figure is assigned
for the potency of each activity in terms of the corresponding activity
of the international standard powder.

APPENDIX

Stehle (1944) has recently drawn attention to the high tryptophane content of melanophore hormone extracts and has proposed an assay of them based on the estimation of tryptophane by the method of May and Rose (1922). About 5 mg. of purified extract is needed for such a determination, and it could only be used for highly purified pituitary extracts.

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Gonadotropins

By CHRISTIAN HAMBURGER

CONTENTS

I. HISTORICAL INTRODUCTION

The presence in the anterior lobe of the hypophysis of hormones possessing specific gonad-stimulating effects was definitely established in the years 1926 to 1927 through the outstanding investigations of Smith and Engle (1927) and Zondek and Aschheim (1927). It is true that before that time several clinical and experimental observations had pointed to the significance of the hypophysis for the functioning of the gonads, but it was not until the above-mentioned publications that the ability of hypophyseal extracts to produce a precocious sexual development in immature male and female animals was demonstrated. The Third International Conference on Standardization of Hormones held in Geneva in 1938 adopted the term: *gonadotropin (-phin)* or *gonadotropic (-phic)* hormones for the active substances which hitherto had been mentioned by several different names.

The gonadotropic action of chorionic tissue was demonstrated in 1927–1928 by the Japanese investigators Murata and Adachi (1928) independently of Aschheim and Zondek's discovery that the blood and urine of pregnant women contain large amounts of gonadotropic hormones, later designated as *chorionic gonadotropin*. The excretion of hypophyseal gonadotropin in the urine of castrated persons was discovered by Aschheim (1928) in postmenopausal and castrated women and by Hamburger (1931) in castrated men.

Through the outstanding series of investigations of Cole and Hart (1930 and following years) we obtained knowledge of the equine gonadotropin—the hormone present in the blood serum of pregnant mares, usually named "*serum gonadotropin.*"

II. SITE OF PRODUCTION AND CLASSIFICATION OF GONADOTROPINS

The production of gonadotropic hormones is limited to three distinct structures: (1) the chromophilic cells of the adenohypophysis; (2) the syncytical cells in the primate trophoblast (and similar cells in certain testicular and ovarian tumors); (3) the "endometrial cups" in pregnant mares (Cole and Goss, 1943).

Corresponding to the sites of production, the gonadotropins fall into three groups: (1) hypophyseal gonadotropins; (2) human chorionic gonadotropin; (3) pregnant mares' serum gonadotropin. Characteristic differences exist between these groups of hormones.

1. Hypophyseal Gonadotropin

Hypophyseal gonadotropin is in all probability not a single factor. Although final proof has not yet been obtained, many facts support the assumption that it is composed of at least three distinct hormones: (1) a hormone stimulating the growth of follicles in the ovary and the germinal epithelium in the testis—the gametokinetic or follicle-stimulating hormone (FSH); (2) a hormone responsible for the corpus luteum development and stimulating the interstitial testicular tissue—the interstitial cell-stimulating or luteinizing hormone (LH); (3) a luteotropic hormone maintaining the function of corpora lutea—this factor being probably identical with the lactogenic hormone, prolactin.

The proportion of these hormones varies according to the species of animal and the age of the individual, and it may be changed through the chemical procedures used in the extraction and purification of the hormones. Hypophyscal extracts are thus qualitatively inconstant; some are predominantly follicle-stimulating, others are mainly luteinizing.

The gonadotropic hormone present in castrate or postmenopausal urines is of hypophyseal origin. It is predominantly or exclusively a follicle stimulator.

2. Chorionic Gonadotropin

Human chorionic gonadotropin has a more limited biological effect than hypophyseal gonadotropin. In animals deprived of their hypophysis it has no follicle-stimulating effect and no action upon the seminiferous tubules. It produces luteinization of the ovarian thecal and stromal tissue. In most intact animals the administration of chorionic gonadotropin causes changes in the gonads very similar to those produced by hypophyseal gonadotropin. This effect is, however, indirect and dependent upon an interaction with the test animal's own hypophyseal hormones, the production or release of which are stimulated by the treatment. The limited action of chorionic gonadotropin is also evident from the fact that, unless given in extremely large amounts, it has no growth-stimulating effect on the testis of immature birds. Nor has it any follicle-stimulating effect in Rhesus monkeys.

3. Serum Gonadotropin

Pregnant mares' serum gonadotropin is more related to hypophyseal gonadotropin in its biological effects; it exerts full gonadotropic action also in hypophysectomized animals and stimulates testicular growth in birds and follicle development in Rhesus monkeys.

The three types of gonadotropin differ in several other respects.

Whereas hypophyseal and chorionic gonadotropins are excreted in the urine both in normal conditions and after administration, the gonadotropic hormone from pregnant mares does not pass the kidneys at all or only in very small amounts.

Finally, chorionic and serum gonadotropins are characterized by their qualitative constancy. In contrast to hypophyseal gonadotropins they are not altered during the process of purification, the gonadotropic effects of the most purified preparations not differing from those of crude extracts.

The above-mentioned differences between the gonadotropic hormones are of great importance for qualitative and quantitative assay. The most characteristic properties of these hormones are summarized in Table I. It must be emphasized, however, that the features mentioned do not apply to all species of animals.   .

TABLE I

Main Similarities and Differences between Various Gonadotropins

Type of gonadotropin	FSH effect	LH effect	Excretion with urine	Qualitative constancy
Hypophyseal gonadotropin (gland. extr.)	+	+	+	O
Hypophyseal gonadotropin (in castrate urine)	+	O	+	+(?)
Chorionic gonadotropin	O	+	+	+
Pregnant mares' serum gonadotropin	+	+	O	+

The exact chemical nature of the gonadotropic hormones is not known. They are probably glycoproteins (Gurin *et al.* 1940a, b). The most purified preparations of chorionic gonadotropin are claimed to be electrophoretically homogeneous and to possess a biological activity corresponding to more than 6000 I.U./mg. Since no reliable chemical test methods for gonadotropins exist, they can only be assayed biologically.

III. General Principles for the Assay of Gonadotropins

1. Choice of Criteria of Response

Since the target organs for gonadotropic hormones are the sex glands, a *conditio sine qua non* of the assay must be that the test animal is in possession of its gonads. Castrated animals are accordingly ruled out. The effects of gonadotropins are made apparent by morphological changes in the ovaries or testes. These changes may be observed by histological technic, by examination of the organs with a hand lens, or often by the naked eye. Usually the weight of the gonads is increased as a result of gonadotropin treatment.

The structural changes in the sex glands are accompanied by an

increased production of sex hormones (estradiol, progesterone, testosterone), and consequently the accessory reproductive organs are enlarged and exhibit characteristic morphological changes. Both the primary effect upon the gonads and the secondary reaction in the accessory organs (uterus, vagina, seminal vesicles, prostate, etc.) are used as end points of assays. More variable factors are involved when the secondary effects are used as criteria of response, e.g., the ability of the gonad to respond to gonadotropic stimulation by increased sex hormone production and the reaction of the accessory reproductive organs to these hormones.

2. Choice of Test Animals

A. AGE, SEX, ETC.

From a theoretical point of view the assay of gonadotropins ought to be carried out with animals deprived of the organ in which gonadotropin is produced, i.e., the hypophysis, just as estrogenic and androgenic substances are assayed in castrated animals. As a matter of fact, experiments with hypophysectomized animals have been of paramount value for our understanding of the physiological properties of the gonadotropic hormones, and hypophysectomized animals are especially superior to intact animals for the assay of gonadotropins in hypophyseal extracts. The technical difficulties of hypophysectomy and the fact that the completeness of the operation must always be checked by serial sections of the sella turcica region after conclusion of the experiments limit the practical use of these test animals to some extent.

For most practical purposes *intact immature animals* are just as useful for the assay of gonadotropin as are castrated animals for the assay of estrogenic hormones. The production by the hypophysis of gonadotropins in immature animals is limited. It is true that the injected gonadotropins may react upon the hypophysis, directly or more likely indirectly through the mobilized sex hormones, but in most assay experiments which involve a comparison of qualitatively identical preparations (standard and unknown) this interaction between the injected and the endogenously produced gonadotropin does not prevent the assay from being performed.

In most *adult animals* the reproductive organs are not suitable as end points for assay of gonadotropic hormones, because they are already heavily stimulated by gonadotropins. Seasonally breeding animals and animals in which ovulation normally occurs only as a result of copulation (rabbit, ferret, cat) form an exception to this rule; the former may be used as test animals during the period of rest and the latter when kept isolated from the males. The adult female rabbit is a particularly

excellent test object for gonadotropic hormones. The South African clawed toad (*Xenopus laevis* Daud.) seldom ovulates spontaneously when kept in captivity, but as in the case of rabbit ovulation takes place after injection of gonadotropic hormones.

The choice of *species of animals* is wide. It depends above all on the cost and the size of the animal, on the breeding capacity of the species and whether the animals are easy to handle or not.

As to *the sex of the test animal*, both males and females are used. Most gonadotropic assays are, however, carried out on females for several reasons, e.g., the changes in the ovaries (follicle stimulation, corpus luteum formation) are much easier to evaluate than the testicular changes (cf. the difficulties to decide whether an increase of the interstitial tissue is relative or absolute). Furthermore, the reliable and practical technic of examining the vaginal smears has no counterpart in the males.

The most commonly used test animals are mentioned below.

B. FEMALE TEST ANIMALS

Since the elaboration of the Aschheim-Zondek pregnancy test the immature female mouse (about 3 weeks old and weighing 6 to 8 g.) has been widely used in gonadotropin assays. Among the advantages of this animal the following may be mentioned. The morphological changes in the ovaries (follicle growth and corpus luteum development) are so characteristic and conspicuous that they are easily recognized by low power inspection of the slightly compressed fresh ovaries. In this way it is possible to decide whether the stimulation is due to chorionic gonadotropin or to the follicle-stimulating hormone in castrate urine. The animals are inexpensive and easy to breed and handle. The most serious disadvantage is their susceptibility to toxins (they do not stand concentrated glandular and urinary extracts).

The female rat 3 to 4 weeks old is probably the most frequently used test animal. Its higher resistance to toxic extracts together with the larger size of ovaries and uterus form the main advantages over the infantile mouse. A serious drawback is the fact that a safe evaluation of the morphological changes in the ovaries necessitates histological examination. Furthermore the structural changes do not allow of a reliable differentiation between the effects of chorionic and hypophyseal gonadotropins. In cases where this decision is desirable the establishment of dose-response curves is needed.

The effects of gonadotropic hormones in immature guinea pigs have been thoroughly examined by Aron (1932/33), Loeb (1932) and Guyénot *et al.* (1935). The purpose of these investigations was mainly to elucidate

the qualitative aspect of the reactions obtained by treatment with various gonadotropins. According to the quantitatively designed experiments of Hamburger and Pedersen-Bjergaard (1946) the female guinea pig must be regarded as less suitable for standardization of gonadotropins for the following reasons: (1) the average ovarian weight is but slightly increased even by administration of very high doses of hormone; (2) it is absolutely impossible to evaluate the morphological changes in the ovaries with-
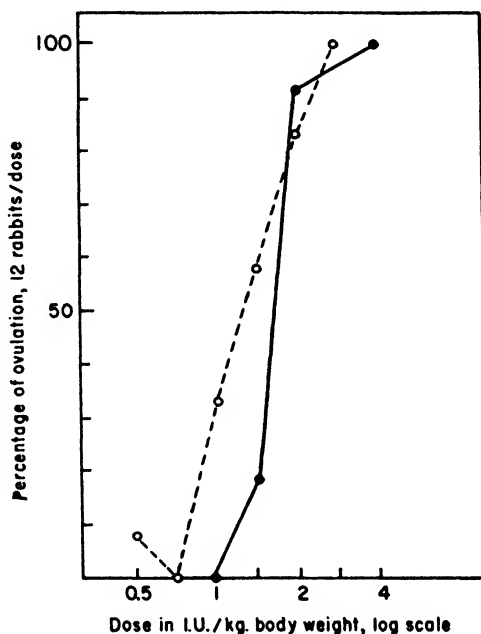
FIG. 1. Comparison of percentage of ovulation obtained in post-partum rabbits (●) and in mature estrus rabbits (○) as a result of intravenous injection of chorionic gonadotropin. (Hormone Dept. of the State Serum Institute, Copenhagen, Dec. 1939.)

out the aid of histological technics; (3) individual variation is very pronounced.

The mature female rabbit is used as test animal in Friedman's pregnancy reaction. Hill et al. (1934) examined the possibility of assaying gonadotropins quantitatively by means of the ovulation test in rabbits and listed the advantages of this method: the end points of assay are well defined ("either ovulation occurs or it does not"); many of the variables encountered in other assay methods are ruled out immediately. The test is of short duration (about 24 hr.) and the animals may be used more than once. According to Friedman (1932, 1939) the individual variation is greatly diminished by the exclusive use of post-partum rabbits

for gonadotropic assay; it was stated that each post-partum rabbit might be expected to afford as much information as 9 to 36 isolated females. Hamburger (1939, unpublished) obtained somewhat steeper dose-response curves (per cent ovulation) when post-partum rabbits were injected with chorionic gonadotropin than when ordinary estrus rabbits were used (Fig. 1), but the difference was not large enough to justify the employment of this more expensive and rare animal. The disadvantages of rabbits as test animals are the higher price, the space they require, and the fact that the type of response is the same irrespective of the type of gonadotropin injected.

The female *Xenopus laevis* has been used for quantitative assay of gonadotropins by Bellerby (1933).

C. MALE TEST ANIMALS

Immature male rats are the only male mammals which have been employed to any extent as test animals for gonadotropic assay. The criteria of response are the weight increase of the seminal vesicles or of the prostate. The advantages of this technic compared with the assay on female rats are not very obvious. Hypophysectomized immature male rats are used in the assay of hypophyseal extracts.

The testes of young male birds are very sensitive to gonadotropic hormones from the hypophysis and to pregnant mares' serum gonadotropin, whereas chorionic gonadotropin is inactive or weakly active. The weight of the testes of young ducks, pigeons, and cockerels and the comb growth of the last-mentioned animals have been used as basis for the assay of hypophyseal and serum gonadotropins, but the methods are more suitable for a qualitative than for an exact quantitative assay.

During recent years attention has been called to the employment of male toads (*Bufo*), frogs (*Rana*) and *Xenopus laevis* as test animals for gonadotropic hormones. The test is based upon the observation that sperm cells appear in the urine a few hours after the subcutaneous injection of gonadotropic hormones. It was proposed as a pregnancy test by Galli Mainini (1947). Robbins *et al.* (1947) state that the male *Xenopus laevis* is a very satisfactory test animal for both hypophyseal and chorionic gonadotropin. Quite recently, Schockaert *et al.* (1948) have assayed the gonadotropins on several species of *Bufo* and *Rana*. The attempts to perform the test quantitatively were encouraging. The toads were found to be more sensitive than the frogs.

3. Complicating Factors

The assay of gonadotropic hormones is complicated by some factors which modify their action, viz., the synergism which exists between

certain gonadotropins, the augmentation of the gonadotropic effect exerted by non-hormonal substances, and the instability of gonadotropic solutions.

A. SYNERGISM

Synergism may be defined as an intensified gonadotropic stimulation obtained when two gonadotropic hormones are administered simultaneously. The reaction of the target-organ is much larger than that which would result from a simple addition of effects. Hypophyseal FSH and LH and chorionic gonadotropin and FSH, e.g., from castrate urine, act synergistically. The phenomenon takes place whether the hormones are mixed before the injection, or are injected separately at different sites.

B. AUGMENTATION

Augmentation of the gonadotropic effect occurs when unspecific substances such as egg albumin, casein, chlorophyll, or lemon juice are mixed with gonadotropic hormones. It is dependent upon a delayed absorption and does not take place when the hormone and "the augmentor" are injected separately.

C. INSTABILITY OF SOLUTIONS

The instability of aqueous solutions of gonadotropic hormones can likewise be an important factor influencing the assay result. The storage of the solutions at temperatures of about 4°C. does not always prevent inactivation. Maddock and Heller (1947) observed a partial inactivation of rat hypophyseal gonadotropin during 3 days' storage at 3°C. Chorionic gonadotropin and serum gonadotropin are, however, more stable than hypophyseal gonadotropin.

D. VARIATION IN SENSITIVITY OF TEST ANIMALS

Variation in the sensitivity of the test animals plays, of course, the same role in the assay of gonadotropins as in other biological measurements. Observations indicate that the mature rabbits are least sensitive to gonadotropic stimulation during the winter months, but in the other commonly used laboratory animals seasonal variations do not occur, or only in a slight degree.

It is worth mentioning, however, that within a breeding colony the sensitivity of the animals can, for reasons still obscure, vary in the course of months or years. In the rat colony of the State Serum Institute, Copenhagen, we have observed that the average ovarian weights obtained in 26- to 28-day-old rats treated with chorionic gonadotropin have

changed in the course of 6 years, •whereas the uterine weight response
has been more constant (see Fig. 2).   Such findings show the necessity
for using standard preparations.

Differences in the response of animals from different laboratories
render it very difficult to give general rules as to which methods of assay
must be regarded as preferable.   This fact is exemplified by the following
observation.   The rats from the breeding colony at the National Institute
for Medical Research, London, react to chorionic gonadotropin in a
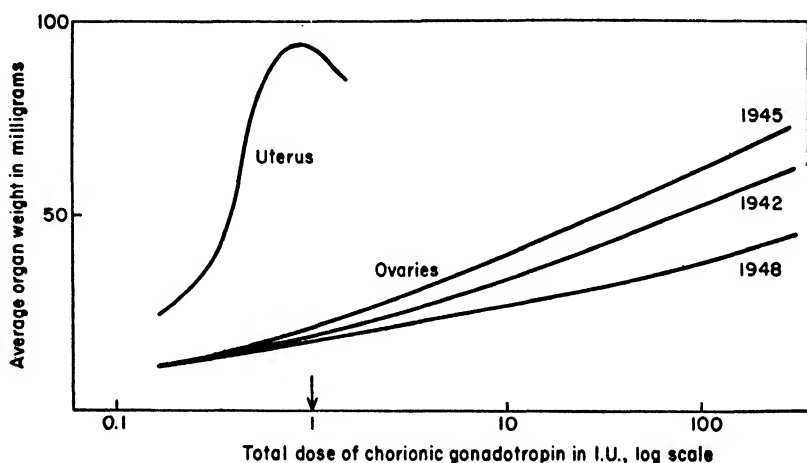
Fig. 2.   Average uterine and ovarian weight curves obtained in immature rats
as a result of treatment with chorionic gonadotropin in the years 1942, 1945, and
1948.   (Hormone Dept. of the State Serum Institute, Copenhagen.)

manner which allows of the use of the ovarian weight curve as end point
of assay, because this curve is sufficiently steep even for comparatively
small doses of hormone (Deanesly, 1935).   The corresponding curve
obtained at the State Serum Institute (Fig. 2) is so flat that an exact
assay can be carried out only when the doses are given over a wide range.

This finding, which is not an exceptional case, is important when
predicting limits of error.   Statements such as "the minimal limits of
error to be expected from an assay" have no absolute validity; even
within the same strain of test animals the "limits of error" may change
in the course of months or years.

The assay of gonadotropic hormones presents a rather intricate
problem.   No wonder, therefore, that cooperative assays carried out
in different laboratories often give inconsistent results, even if the assays
are made with a standard preparation and an unknown preparation
which must be considered qualitatively identical.   It is not the intention

here to give anything like a complete survey of the innumerable publications on methods for the assay of gonadotropic hormones found in the world literature. We shall confine ourselves to mention and discussion of the most commonly used methods of assay. Within each section the assay for clinical purposes and the assay of more purified (commercial) preparations will be described separately.

IV. Assay of Chorionic Gonadotropin

1. International Standard Preparation

At the Third International Conference on the Standardization of Hormones, held in Geneva in August, 1938, under the auspices of the League of Nations, it was decided to establish an international standard for the gonadotropic substance of human urine of pregnancy. The Conference had before it the results of a cooperative examination of the various preparations offered toward the proposed standard, and the members agreed upon the details of the manner in which the preparations should be mixed. Subsequently the material was diluted with lactose and dried and was then made into tablets of approximately 10 mg.

The unit was defined as follows:

"The specific gonadotropic activity of 0.1 mg. (= 100 μg.) of the standard preparation shall be the international unit for the activities of all gonadotropic preparations of human urine of pregnancy, *but only of such.*" The standard is kept at the National Institute for Medical Research, London and is distributed therefrom to national control centers and to other research institutes.

It was agreed "that the only tests for the comparative determination of gonadotropic activity, in units as above defined, on which the Conference has evidence to justify recommendation, depend on:

(a) The observation of a direct or indirect gonadotropic effect, shown by morphological changes in the gonads; or (b) The observation of secondary changes in the accessory reproductive organs in animals not deprived of their gonads. When this type of test is used, the absence of substances directly causing such changes in the accessory reproductive organs should be assured by control tests on animals deprived of their gonads." The standard was established, and the unit brought into current use on April 1, 1939. (Memorandum: *Bull. Health Org.* 1939, **8**, 884.)

In the course of the decade during which the standard has been established, it has been of very great value for clinical hormone analyses, for the production of commercial preparations, and for the control of the preparations on the market. The practical usefulness of the standard

supports the assumption that all chorionic gonadotropic preparations are qualitatively constant, and so far no convincing observations have indicated the existence of such qualitative differences as would make the use of the standard impossible.

2. Clinical Analyses with Urine

The determination of chorionic gonadotropin in the urine or blood is of high importance for the diagnosis of pregnancy, hydatiform mole and chorionepithelioma, and also for certain malignant testicular and
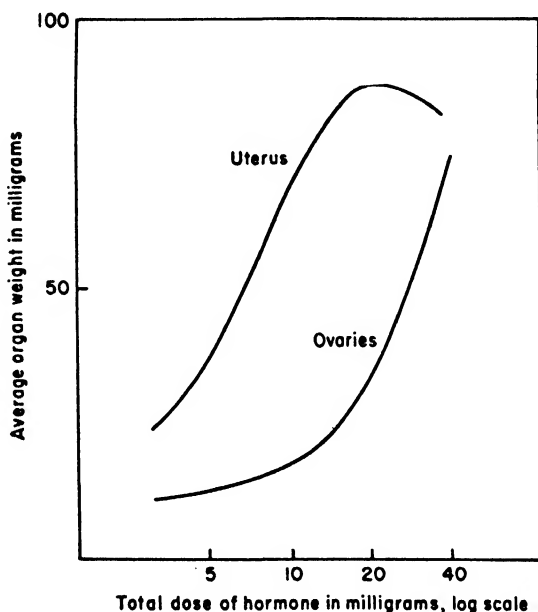
FIG. 3.   Average uterine and ovarian weight curves obtained in immature rats after treatment with FSH from postmenopausal urines.   (Hormone Dept. of the State Serum Institute, Copenhagen.)

ovarian tumors.   In many cases a reliable qualitative determination of the type of gonadotropin found is highly desirable.   For instance, a weakly positive Friedman test obtained with the urine of a woman in the late forties may be due to an early pregnancy (chorionic gonado-tropin), or to an increased excretion of FSH (hypophyseal gonadotropin) as a result of a beginning climacteric.   In cases of malignant tumors of the testis the gonadotropin can be of hypophyseal origin, probably as a consequence of a decreased androgen production, or the hormone can be chorionic gonadotropin produced by trophoblast-like cells in the tumor tissue.   Also after the removal of a hydatiform mole it is important to

decide whether a gonadotropic reaction is due to the presence of chorionic tissue or to hypophyseal gonadotropin (impaired ovarian function, e.g., in cases of lutein cysts).

This qualitative assay can be carried out in several ways; a convenient and reliable test object is the immature mouse ovary. Female mice, 21 days old are injected with untreated urine or dilutions thereof, or extracts of tannic acid or alcohol precipitated urine, for 2 days. On the fifth day the ovaries are dissected and examined by a hand lens under slight compression between a black glass plate and a slide. Ovaries
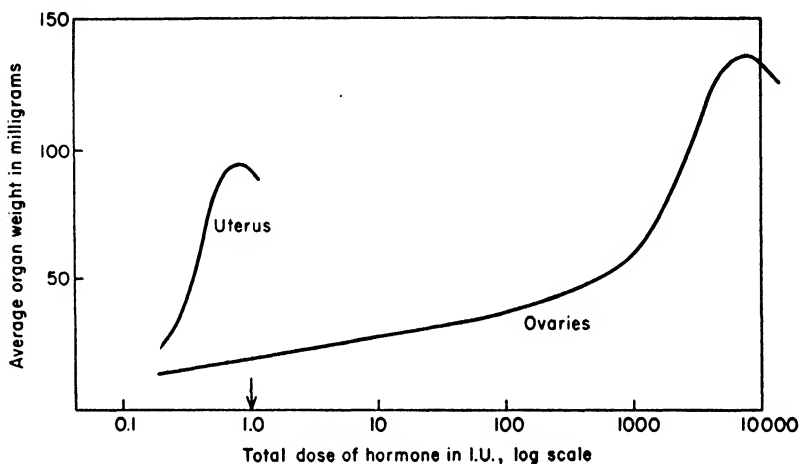
Fig. 4. Average uterine and ovarian weight curves obtained in immature rats after treatment with chorionic gonadotropin. (Hormone Dept. of the State Serum Institute, Copenhagen.)

stimulated by FSH are pale and contain numerous medium-sized follicles but no corpora lutea or hemorrhagic follicles, whereas ovaries stimulated by chorionic gonadotropin are hyperemic and contain corpora lutea and/or "blood points" and solitary large follicles.

In immature female rats the shape of the logarithmic ovarian dose-response curves and their relation to the uterine curve differ so much in rats treated with FSH and with chorionic gonadotropin that qualitative analyses can be performed safely in this way (see Figs. 3 and 4).

When a quantitative determination of the content of chorionic gonadotropin is desired, e.g., for the diagnosis of hydatiform mole, the gradual rise in the rat's ovarian weight curve has some advantages. In these instances the result of the analysis must be available within a comparatively short time and the urine may contain up to 20 million I.U. of chorionic gonadotropin per liter. While otherwise a steep curve is

advantageous, in these cases a steep curve is a drawback because it will cover only a narrow range of dosage. When groups of two rats are treated with 5, 1, 0.1, 0.01, 0.001, and 0.0001 ml. of urine, the content of chorionic gonadotropin in the urine can be determined in the course of 4 days, if it lies between 40 I.U. and 10 million I.U. per liter. Practical experience has shown that this assay is sufficiently accurate for clinical use (Hamburger, 1943).

The abrupt rise appearing in response to doses from about 1000 to about 10,000 I.U. of chorionic gonadotropin has given rise to various hypotheses. One may suppose that the rise is caused by contamination with hypophyseal gonadotropin and that this substance would exert its influence with the high doses. This explanation is, however, not very probable, since the rise appears in about the same place in the curve in spite of the fact that the gonadotropin may be derived from the urine of pregnant women, from patients with testicular tumors or from cases of chorionepithelioma. The two hormones should, according to this assumption, be present in an approximately constant ratio in all three cases. The fact that chorionic gonadotropin probably produces its effect in intact animals partly by stimulation of the test animals' hypophyses is of no importance in this connection. Pertinent data and discussion are found in papers by Evans (1936), Fevold et al. (1939) and Hamburger (1941).

All the other methods of assaying commercial preparations of chorionic gonadotropin, listed below, are, of course, applicable for the assay of the hormone in the urine, blood, and tissue extracts. When changes in the secondary reproductive organs are used as end points of response the possible interfering effect of estrogenic substances present in the fluids must naturally be considered.

3. Assay of Commercial Chorionic Gonadotropin Preparations

The demands of accuracy are greater when dealing with the assay of commercial preparations than in the case of clinical analyses, whereas the time required for the analysis is of minor importance. A great number of publications have appeared on the accuracy of the various methods of assaying chorionic gonadotropin.

Shortly after the Third Conference on the Standardization of Hormones, Emmens (1939a) published a report containing an analysis of the cooperative assays carried out on the preparations offered as contributions to the standard. The great majority of the assays had been carried out on immature female rats, but immature male rats, immature female mice, adult female rabbits and male ring-doves were also used as test animals. The following kinds of changes were used as assay end

points: The increase in ovarian or uterine weight, vaginal cornification, the formation of corpora lutea, increase in weight of the prostate glands and/or the seminal vesicles in male rats, the production of ovulation in rabbits, and increase in the weight of the testes in ring-doves.

A statistical analysis of the results showed that the vaginal cornification test in immature female rats was the most accurate test; furthermore it is the most labor-saving and inexpensive test method. In respect to accuracy the vaginal smear technic was followed by the method using ovarian weight as the end point of response. This method and the assays based upon the corpus luteum formation were recommended, partly in view of their reasonably high accuracy, partly because they are independent of the presence of estrogenic substances in the preparations. The uterine weight determinations were found to be less satisfactory than the vaginal smear technic, but they were recommended as a means to check the results of the latter method.

The test methods did not influence the determination of the relative activity of the preparations tested, a result which supports the view that there is only one gonadotropic substance in pregnancy urine. The different preparations were, however, assayable with significantly different degrees of accuracy. This finding, together with the fact that the variance between estimates from different laboratories was significantly greater than that between estimates by different test methods from the same laboratory, indicated that some of the preparations, diluted with lactose, were not homogeneously mixed.

An appendix to Emmens' report (1939a) contains a description of the methods of assay used by the members of the Conference. It was a common feature of all methods that the total dose was divided into several doses distributed in the course of from 2 to 5 days, with the exception of the rabbit ovulation test in which only one intravenous injection was given. The result of assay varies greatly with the mode of administration, a single subcutaneous or intravenous injection giving much weaker reactions (Hamburger and Pedersen-Bjergaard, 1938).

Space does not permit a detailed description of the almost innumerable variations in the technic of assay. Hence only a few examples will be mentioned as prototypes of the assay methods.

A. VAGINAL CORNIFICATION IN RATS

Groups of 10 or 20 immature rats are used, 21–28 days old. The total dose of the preparation to be tested is dissolved in water or saline and given by 5 equal subcutaneous injections over a period of 48 hr. Vaginal smears are taken on the fourth to the sixth day after the first injection. The criterion of response is the completely cornified vaginal smear. The

log dose-response curve (percentage of animals showing positive reaction) is S-shaped and covers, in most strains of rats, the range of dosage from 0.25 to 1.0 I.U.

B. UTERINE WEIGHT IN RATS

The rats are selected and treated as above. About 96 hr. after the first injection the animals are killed and the uteri are removed by cutting at the utero-cervical and at the salpingo-uterine junctions, then the mesometrium is torn from the uterus and the fluid drained from the uterine cavity before weighing. The S-shaped dose-response curve usually covers the range of dosage from 0.125 to 1.0 I.U. Dorfman and Rubin (1947) express the responses as the ratio of the uterine weight in milligrams to the body weight in grams. They use 4 groups of rats, 2 groups receiving the standard and 2 the unknown. The doses are so chosen that the ratio of the high dose to that of the low dose is the same for both the standard and the unknown. The relative potency, the mean slope, the standard deviation, the error range, and the significance of difference of the respective slopes are calculated. Delfs (1941) uses 21- to 23-day rats and gives 6 equal injections in the course of 2 days. The animals are killed 72 hr. after the start of injections as the 72-hr. uterine weight curve was found to be more sensitive and more responsive to increments of dosage than the uterine weight at 96 hr. Several authors (among whom D'Amour and D'Amour, 1940) using the uterine weight method combine this technic with vaginal smear examination.

C. OVARIAN WEIGHT IN RATS

As mentioned above, not all strains of rats are suitable for this method of assay. The weight curve obtained with the rats in the State Serum Institute, Copenhagen, is too flat to allow of an exact assay (cf. Fig. 4). The same seems to be the case with the immature rats available in several other laboratories, the curves shown in a paper of Evans (1936) resemble those from Copenhagen; Laqueur in Amsterdam (Emmens, 1939a) stated that the ovarian weight was not significantly changed by 12 I.U., and D'Amour and D'Amour (1940) obtained merely a doubling of the average ovarian weight by 10 I.U. The rats at the National Institute for Medical Research, London, are, however, very suitable for this technic. This fact is evident from the papers by Deanesly (1935) and Rowlands (1935) and, according to Emmens (1939a) 12 to 15 I.U. cause a fourfold increase in ovarian weight. The British Pharmacopoeia (1948) recommends the method, performed in this way. Rats weighing from 40 to 50 g. are treated with one daily subcutaneous injection for 5 days and killed 24 hr. after the last injection. The ovaries are cut

free and fixed in solution of formaldehyde and trinitrophenol overnight and immersed in 70% alcohol for between 2 and 4 hr. They are then freed from the remaining hilum and weighed on a torsion balance after drying for a constant short period of time on filter paper. Four groups of rats, not less than 10 in each group, are required for the final assay. The rats in the first group receive a total dose of the standard expected to produce a mean ovarian weight of 15 to 20 mg., in the second group, 30 to 35 mg. The rats in the remaining 2 groups receive corresponding doses of the preparation being tested, the approximate potency of which has been tested in a pilot assay. The results are subjected to an analysis of variance and are expected to give limits of error ($P = 0.99$) of 80–125% if littermates are used, but only 65–155% with random animals.

The advantage of fixing the organs and weighing them from 70% alcohol is, however, not obvious, on the contrary, the evaporation must be a more disturbing factor than when weighing the fresh organs.

D. CORPUS LUTEUM FORMATION IN MICE

Groups of 10 to 20 mice, 21 days of age, are treated with 5 equal subcutaneous injections in the course of 48 hr. The animals are killed about 96 hr. after the first injection; the ovaries are dissected and the formation of corpora lutea observed by examination of the slightly compressed ovaries with a lens. The criterion of response is the number of ovaries showing corpus luteum formation expressed as a percentage. The range of dosage covered by the dose-response curve extends from 1 to 8 I.U. (Hamburger and Pedersen-Bjergaard, 1937).

E. OVULATION IN RABBITS

Adult female rabbits, isolated from males for 3 to 4 weeks, or postpartum rabbits, used 2 to 25 days after parturition of a litter of at least 4 young, are treated with a single intravenous injection of the preparation dissolved in water or saline. The animals are killed, or the ovaries inspected by laparotomy, 24 to 40 hr. afterwards. The criterion of response is ovulation (Hill *et al.*, 1934, and Friedman, 1932, 1939). The animals may be used again after a lapse of 3 to 4 weeks, more than three tests on the same animal are, however, not to be recommended.

F. REPAIR OF OVARIAN "DEFICIENCY CELLS" IN IMMATURE HYPOPHYSECTOMIZED RATS

The method is advocated by Evans (see Emmens, 1939a) and is carried out in this manner: 25- to 28-day-old hypophysectomized rats receive 3 daily subcutaneous or intraperitoneal injections, followed by autopsy at 72 hr. from the first injection. The postoperative interval

is 8 to 10 days, and the criterion of response is the repair of the characteristic nuclear pattern of the ovarian interstitial cells ("deficiency cells").

V. Assay of Pregnant Mares' Serum Gonadotropin

1. International Standard Preparation

The establishment of the international standard preparation for this hormone was recommended at the Third International Conference on the Standardization of Hormones in Geneva, 1938. Samples of this substance were afterwards distributed among experts, who found them all suitable for inclusion in the standard preparation. The specimens were mixed and diluted with lactose and again assayed by the experts, including members of the Conference. It was agreed that the specific gonadotropic activity of 0.25 mg. (= 250 μg.) of the standard preparation should be the international unit for recording the activities of all gonadotropic preparations of the serum of pregnant mares, *but only of such*. The Conference recommended the following use of the standard in biological assay: the observation of a direct or indirect gonadotropic effect, shown by morphological changes in the gonads; or the observation of secondary changes in the accessory reproductive organs, in animals not deprived of their gonads (with control tests for estrogenic substances as for chorionic gonadotropin, p. 183).

The standard preparation was made into tablets of approximately 25 mg. and is stored at the National Institute for Medical Research, London, and distributed therefrom to national control centers for the control of such substances, and to other suitable research institutes. (Memorandum: *Bull. Health Org.* 1939, **8**, 898.)

The standard and unit came into force in November, 1939, and since then most commercial serum gonadotropin preparations are labeled in I.U. No difficulties for the practical usefulness of the standard preparation have been observed, a fact which supports the view that the hormone in untreated serum and that in the highly purified preparations are qualitatively constant (see below).

2. Clinical Analyses with Serum

The determination of serum gonadotropin is the basis of a pregnancy test in the mare (Cole and Hart, 1930). These authors found the concentration of the hormone in the blood sufficiently large to allow of the assay from the 42nd to the 180th day of pregnancy. Day and Rowlands (1940) confirmed the statements of Cole and Hart as to the appearance of the hormone in the blood and the time of maximal concentration. They found, however, a more rapid disappearance of the

hormone (by the 110th day). Glud *et al.* (1933) obtained a positive Aschheim-Zondek reaction from the 42nd to the 136th day of pregnancy. The commonly used end point of assay is now the increase in the ovarian weight of immature rats or mice. The serum is given subcutaneously once, or the total dose may be divided into 3 or 5 equal doses. The total dose amounts to 1 or 2 ml. for mice and up to 5 ml. for rats, and the animals are autopsied 72 or 96 hr. after the beginning of the experiment. As the gonadotropic hormone present in pregnant mare serum is not excreted with the urine, or only very small quantities, the analysis cannot be carried out with urine.

3. Assay of Commercial Serum Gonadotropin Preparations

Emmens (1939b) analyzed the cooperative assays carried out in various laboratories on the preparations offered toward the international standard preparation. Most of the experts had used immature female rats as test animals; male rats, immature female mice, and adult rabbits were used by some investigators. The criteria of response included the following kinds of changes: increase in ovarian weight, increase in uterine weight, vaginal cornification, formation of corpora lutea, increase in the weight of the seminal vesicles, and ovulation in rabbits. The tests using ovarian weight as an end point of assay were more accurate than the miscellaneous tests using other criteria of response considered together, but these other assays were not performed in sufficient numbers to be considered separately from the rest.

The superiority of the rat ovarian weight method had been demonstrated by several authors before the establishment of the standard. Hamburger and Pedersen-Bjergaard (1937) examined a commercial preparation (Antex Leo) on immature female mice and rats using several different changes as criteria of response. They found that the steep part of the rat ovarian weight curve was the best end point of assay. Figure 5 shows the result of the experiments with rats. Twenty rats were used for each dose. The total doses were given as 5 equal injections in the course of 48 hr. and autopsy was performed on the sixth day after the first injection. The smaller doses produced a steep increase in average uterine weight; a 32-fold increase of the dose brought forth a very steep increase in the ovarian weight. The average weight of ovaries had a maximum at 150 mg., i.e., 15 times that of untreated controls. The formation of corpora lutea was stated to be irregular; cornification of the vaginal smear occurred when the doses were somewhat larger than those producing the uterine weight increase.

Similar results were obtained by Cartland and Nelson (1938) in experiments with immature female rats. A 15 times increase in the

mean ovarian weight was produced by increasing the dose of hormone to 15 times the minimal effective dose. Luteinization was found to be a regular function of dosage—corpora lutea of ovulation appeared at small doses, somewhat higher amounts having a purely follicle-stimulating effect, while still higher quantities produced atretic corpora lutea. It is interesting to compare these results obtained in Kalamazoo, Michigan (Fig. 6), with those from Copenhagen, Denmark. The shape of
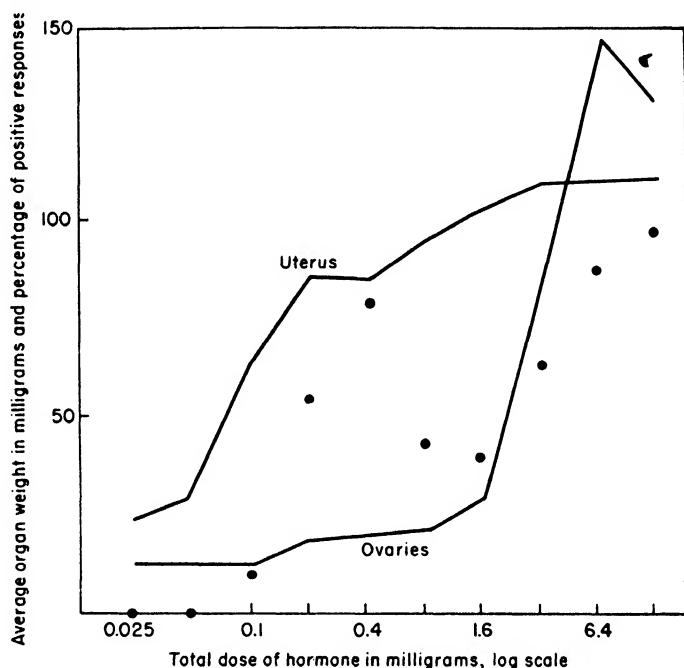
FIG. 5. Response of uterus and ovaries of immature rats to pregnant mares' serum gonadotropin. The black circles indicate the percentage of ovaries with corpus luteum formation. (After Hamburger and Pedersen-Bjergaard, 1937.)

the uterine and ovarian dose-response curves is alike and the two-peak curve for the percentage of luteinization is evident in both experiments, although less pronounced in the Danish investigation.

The identity of the gonadotropic properties of the original crude plasma and the purified preparations has been stressed by Cartland and Nelson (1937), Hamburger and Pedersen-Bjergaard (1937), Rowlands (1938), Hamburger (1938), Emmens (1939b), and Cole *et al.* (1940). The last-mentioned investigators compared the effects of native plasma with those of a purified preparation containing 3000 to 4000 rat units/mg., in experiments with intact as well as hypophysectomized female rats. These widely different specimens had identical biological effects. If

the follicle-stimulating and the luteinizing effects of pregnant mares' serum gonadotropin were due to two distinct hormones, these must be present in the same ratio in all the preparations, and this is hardly probable. Some older reports claiming the separation of the two hor-
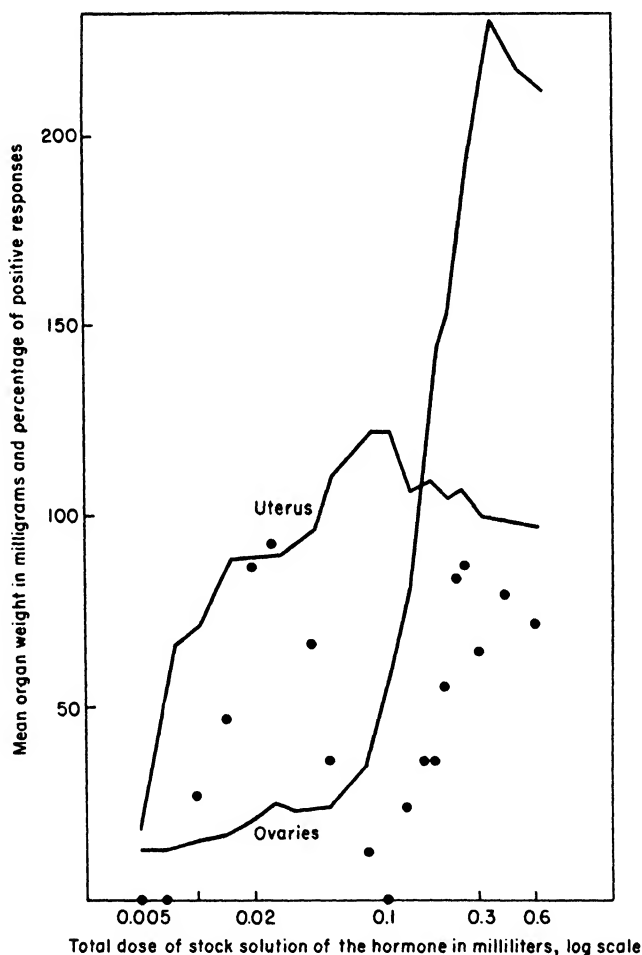
FIG. 6. Response of uterus and ovaries of immature rats to pregnant mares' serum gonadotropin. The black circles indicate the percentage of ovaries with corpus luteum formation. (After Cartland and Nelson, 1938.)

mones in pregnant mares' serum are not very convincing and were partly abandoned by the authors themselves in later publications. Quite recently, Aldman *et al.* (1949) in experiments on the estrin precursors in the ovaries of rats have tried a very ingenious method of separating

the FSH and LH in serum gonadotropin taking advantage of the fact that injected hypophyseal LH is rapidly eliminated from the blood, while elimination of the serum gonadotropin itself occurs slowly. A female rabbit was spayed and hypophysectomized, and three weeks later a large quantity of a commercial serum gonadotropin was injected intravenously. Four days after the injection, when a contaminating LH must have almost entirely disappeared from the blood, the animal was killed and its serum was precipitated with acetone and dried. The authors claim that the preparation, purified in this biological manner, differed from the original preparation in respect to its effect on the estrin precursor in the ovaries, and that the difference might be explained through the elimination of LH. It is desirable that the investigations be extended to a study of the follicle-stimulating and luteinizing properties of such preparations.

As far back as 1932, Cole, Guilbert, and Goss found that serum gonadotropin was just as effective when administered in a single dose to 25-day-old rats as when the dose was divided into 8 injections over a period of 4 days. This observation was confirmed by Hamburger and Pedersen-Bjergaard (1938) who compared the effect of repeated and single subcutaneous, and single intravenous injections of serum gonadotropin and chorionic gonadotropin in immature rats. The effect of serum gonadotropin, but not of chorionic gonadotropin, was quite independent of the mode of administration. Since the effect of serum gonadotropin is not augmented by such substances as augment other gonadotropins (Saunders and Cole, 1936; Lein, 1937) it is possible that this hormone is absorbed slowly from the tissues. This slow absorption rate must, however, be of minor importance as intravenous injections were as effective as subcutaneous injections. It is reasonable to assume that a slow destruction of the hormone in the blood stream (Catchpole et al., 1935), coupled with the non-excretion is the reason for the independence of results with serum gonadotropin of the mode of administration.

In view of this fact it is rather surprising that most investigators prefer to inject the amount of hormone as 3 or 5 subcutaneous doses. In the cooperative assay of the contributions to the standard (Emmens, 1939b) all the experts used the divided dosage technic.

Some examples of the commonly used methods of assay are listed below.

A. OVARIAN WEIGHT IN RATS

Groups of 10 to 20 immature rats are injected subcutaneously with the standard and with the unknown preparation. The total dose is usually

given in 3 or 5 doses over 2 or 3 days.   About 96 hr. after the beginning of the experiment the animals are killed and the ovaries dissected free from bursae and fat, then blotted on moistened filter paper and weighed. The average ovarian weights usually fall on the steep part of the dose-response curve when the total dose amounts to from 8 to 32 I.U.

The British Pharmacopoeia (1948) warns against the use of that part of the rat ovarian curve which is above 100 mg.   The 2 doses of the standard and of the preparation to be tested are so chosen that the expected mean response to the lower dose is 40 to 50 mg., and to the higher dose 80 to 100 mg.   The total dose of hormone is given in a single subcutaneous injection in 1 ml. of water, and the groups of 10 rats are killed on the sixth day of the test.   The results of assay are subjected to an analysis of variance and are expected to give limits of error ($P =$ 0.99) of 90–110% when litter-mates are used, but only of 80–125% with randomly chosen animals.

Cole and Erway's 48-hr. assay (1941).   Groups of 6 immature rats are injected once with doses from 4 to 20 I.U. and the ovaries are dissected and weighed 48 hr. later.   Although the average ovarian weights are lower when the animals are autopsied after 48 hr. than after 72 or 96 hr., the difference between responses dependent upon dosage was found to be as significant at 48 hr. as it was at longer intervals.   The determination of the activity of the unknown preparation is made by means of a regression line calculated by the method of least squares on the basis of the average ovarian weights obtained by administration of the standard preparation.

Albert and Money (1946) have proposed a method of assay based on the rate of ovarian growth in rats.   The hormone is given to 21-day rats as a single subcutaneous injection and one half of the animals is killed 24 hr. later, the other half 48 hr. after the injection.   The rate of ovarian growth is calculated from the average ovarian weights.   The variation in determining the rate is stated to be less than that encountered when using only the 48-hr. reading.

The only serious drawback to the above-mentioned methods of assay is the large quantity of standard required.   Not infrequently an amount up to 1000 I.U. is necessary for a single assay.   National standard preparations or laboratory substandards must therefore be established and the international standard saved for the assay of these substandards.

The ovarian weight method can also be carried out on immature female mice.   The necessary amount of hormone is lower; total doses from about 2 to 10 I.U. may be expected to produce ovarian weights falling on the steep part of the curve.

B. UTERINE WEIGHT IN RATS

When the amount of substance is limited, the assay can be based on the average uterine weights. The hormone is administered as stated above, but the optimal doses are much smaller. Usually the total dose has to be from 0.25 to 1.0 I.U., depending on the sensitivity of the rats, as this range of dosage covers the steep part of the uterine weight curve.

VI. Assay of Hypophyseal Gonadotropins

1. International Preparation

At the Third International Conference on the Standardization of Hormones, held in Geneva in 1938, it was unanimously agreed that the Conference was unable to recommend the establishment of an international standard preparation for measurements of units of the gonadotropic effects of preparations from the anterior lobe of the hypophysis. With a view to facilitating research, and particularly the comparative assay of the hormones of the anterior lobe of the hypophysis, for which international standard preparations has not yet been adopted, the Conference found the establishment of an international preparation of dessicated ox anterior pituitary gland desirable.

The National Institute for Medical Research, London, undertook the arrangement of the preparation, and from 1942 it has been available for distribution to the national control centers or to institutes or research workers. The preparation consists of 4.5 kg. of dessicated ox anterior pituitary gland in the form of a finely divided powder; it is filled out into ampoules, each containing approximately 5 g.

The gonadotropic and thyrotropic activity of the preparation together with the prolactin content have been tested at the National Institute for Medical Research, London, and in some other laboratories. The results of these assays are published in a memorandum, October, 1942 (*Bull. Health Org.* 1942/43, **10**, 94).

2. Assay of Hypophyseal Extracts

As mentioned above, it is likely that 3 distinct gonadotropic hormones are produced by the anterior lobe of the hypophysis, viz., the follicle-stimulating hormone (FSH), the luteinizing hormone (LH), and a luteotropic hormone. The effect of the last-mentioned hormone seems to be the maintenance of the corpora lutea and of progesterone production by these structures. It is probable that the hormone is identical with the lactogenic and crop-sac stimulating hormone (prolactin). The assay of this substance is treated in another chapter of this volume.

Fevold and co-workers have published several papers on the separation of the FSH and the LH in hypophyseal extracts and have described the methods of assay of these substances (Fevold, 1937; Fevold *et al.*, 1937; Fevold, 1939). It is emphasized that the assay of hypophyseal gonadotropin is very difficult, since the reaction obtained will depend on the proportion of FSH and LH present in the extract. If an extract contains a large amount of FSH and only a small amount of LH, the effect of the latter may be suppressed. When FSH alone is given to rats the increase of the ovarian weight obtained is limited. In experiments of short duration the enlarged size of the ovaries is due exclusively to growth of the follicles. By simultaneous injections of FSH and LH the ovarian weights increase to much higher average values than those obtained by FSH alone, the two hormones acting synergistically. The LH alone does not increase the weight of the ovaries.

Fevold's method of assay of an unfractionated or of a follicle-stimulating hypophyseal extract is based on the increase of the ovarian weight. Rats, 21 days of age, are injected twice daily, with the extract, for 3 days, and autopsy is performed on the fourth day. One rat unit is defined as the amount of extract which produces from 50 to 100% increase in weight over uninjected controls.

Since the purified LH preparations have no macroscopic effect on the ovaries of immature rats, its assay is accomplished by combining it with FSH. One rat unit of LH is the amount of the substance which, when combined with two rat units of FSH, and injected over a period of 5 days, will produce a 100% increase over that produced by the FSH alone. The luteinizing hormone can also be assayed on 21-day-old male rats. The injections are continued for 5 days, and the seminal vesicles are weighed on the morning of the sixth day. Fevold's unit is the amount which doubles the weight of the seminal vesicles.

D'Amour and D'Amour (1938) found male rats unsatisfactory as test animals in the assay of hypophyseal powder from sheep and preferred the ovarian weight method.

Hill *et al.* (1933) in their work on the rabbit ovulation test defined the unit of an ox pituitary extract as the amount required to cause ovulation in 50% of a group of not less than 10 estrus rabbits, the extract being administered as a single intravenous injection. The authors emphasized the unsoundness of comparing the rabbit unit and the mouse (or rat) unit of an extract. Rowlands (1935) reported that when ox and horse hypophyseal extracts are assayed in rabbit units, 20 times as much of the ox extract than of the horse extract is required to obtain a certain increase in the rat ovarian weight.

Heller *et al.* (1938) working with extracts of rat hypophyses preferred

the uterine weight of immature rats as the end point of the assay. It was found to be about 8 times as sensitive as the ovarian weight method. The ratio of the uterine unit to the ovarian unit and the shape of the dose-response curves obtained differed, however, depending upon the source of the material.

Space does not permit mention of the numerous other methods proposed for the assay of hypophyseal gonadotropins using intact animals as test objects. The synergistic and antagonistic effects of the active substances and the interaction between them and the test animal's hypophysis make them all rather unreliable. For the assay of such qualitatively inconstant preparations immature hypophysectomized animals are preferable as test objects.

H. M. Evans et al. (1939) recommend the use of female rats, hypophysectomized when 26 to 28 days old and used 6 to 8 days later. The rats are injected once daily over a period of 3 days and they are killed 72 hr. after the first injection. The follicle-stimulating hormone which is administered subcutaneously gives rise to the formation of numerous antrum-containing follicles without any sign of luteinization, whereas the luteinizing hormone, given intraperitoneally, causes the disappearance of the "deficiency cells" ("wheel-cells") in the thecal tissue of the ovary.

In immature hypophysectomized male rats FSH produces a weight increase of the testes owing to stimulation of the germinal tissue only. Since this stimulation is not accompanied by any androgen production the accessory reproductive organs remain atrophic. The LH stimulates the growth of interstitial testicular tissue and the production of androgenic substances resulting in an enlargement of the accessory reproductive organs.

According to the interesting investigations of Nalbandow et al. (1946) hypophysectomized cocks are ideal test animals for FSH and LH. Preparations of FSH absolutely free from LH will cause an increase of the testes but no enlargement of the comb. Preparations of LH alone cause both comb growth and enlargement of the testes. The synergism existing between FSH and LH is clearly demonstrated. Chorionic gonadotropin was found ineffective in hypophysectomized cocks.

A chick assay of unfractionated hypophyseal extracts is performed by J. S. Evans et al. (1940) as follows. One-day old cockerels receive two injections daily for 5 days, they are killed 18 hr. after the last injection, and the testes are weighed.

3. Clinical Analyses with Urine

The gonadotropic hormone present in the urine of male and female individuals deprived of their gonads, or suffering from impaired produc-

tion of gonadal hormones (castration, climacteric, senescence) is produced by the anterior lobe of the hypophysis and seems to be identical with the FSH isolated from this gland.

The hormone may occur in the urine in such amounts as allow of a biological assay in the native urine, but usually it is necessary to prepare an extract of the urine before the assay. The hormone can be precipitated by alcohol, tannic acid, or absorbed on benzoic acid, etc.

Experiments with hypophysectomized rats of both sexes have shown that in castrate urine the hormone is a purely follicle-stimulating hormone. It is possible to assay this hormone safely on immature animals, not deprived of their hypophysis, preferably female mice or rats.

Frank and Berman (1941) found that the estrogenic hormones produced by the stimulated ovaries do not influence the test animal's hypophysis in such a degree as would make the test on immature rats unreliable.

The commonly used criteria of response are: (1) the degree of follicle stimulation in the mouse or rat ovary, as determined microscopically; (2) the increase in the weight of uterus or ovaries of immature mice or rats; (3) the production of a cornified vaginal smear; (4) histologically demonstrable changes in the vaginal mucosa of immature rats.

Using the changes in the accessory genital organs as end points of response, one must have in mind that the presence of gonadal hormones in the extracts may influence the result. Palmer (1937) has found that estrogenic substances actually can be present in the extracts prepared by precipitation methods.

The evaluation of the morphological changes in the immature mouse ovary (follicle stimulation, corpus luteum formation) is very reliable from a qualitative point of view, but too subjective to allow of an exact quantitative assay. It is, therefore, desirable to employ a test method which includes both an objective criterion, e.g., ovarian weight, and an evaluation of the structural changes in the ovaries. The assay may be carried out as follows.

Groups of 5 female mice, about 21 days old, receive subcutaneous injections of the untreated urine or extracts hereof. The total amount is divided into 5 equal doses, administered in the course of 2 days. The mice are killed 90–100 hr. after the first injection, and the ovaries are weighed and examined histologically or by inspection under slight compression. One mouse unit may be defined as the amount of hormone which produces a 100% increase in ovarian weight over those of the controls and at the same time a pure follicle stimulation.

For the evaluation of the morphological changes in the rat ovary histological examination is necessary. The Frank-Salmon test (Frank, 1938) for the assay of gonadotropic hormones in urine and blood is

based upon histological estimation of follicle stimulation and corpus luteum formation in immature rats (24–26 g. at the beginning of the experiment).

The method using the mouse uterine weight as a criterion of response has been thoroughly investigated by Levin and Tyndale (1937). Their "mouse uterine unit" is defined as the amount of material which, when administered to each of 5 or more 21- to 23-day-old mice in 3 equal portions at 24-hr. intervals, on the fourth day (72 hr. after the first injection) produces 100–150% increase in average uterine weight. The authors found the assay of FSH in castrate or post-menopausal urine on the basis of uterine weight to be at least 3 times as accurate as that based on ovarian weight, and far more sensitive.

Ovarian and uterine weight curves obtained in immature rats are widely used for the assay of FSH in urine. Usually, groups of 5 female rats, 26–28 days old are injected with 3 or 5 equal doses of the urine extract for 2 or 3 days. Autopsy is performed on the fifth day and the uterus and/or the ovaries are weighed. From the dose-response curves the average weights obtained may be transformed into defined rat units.

Histological examination of the vaginal mucosa. Thomsen and Pedersen-Bjergaard (1936) used the principle in the Fluhmann test for estrogenic substances in their method of assaying gonadotropin in urine. (The Fluhmann test is performed on adult spayed mice and is based on the fact that a histologically demonstrable mucification of the vaginal epithelium occurs as the result of treatment with amounts of estrogens far below those which produce a cornification of the cells.) Adapted for gonadotropin assay the method is carried out as follows. The urine is precipitated with tannic acid, and different doses of the extract are injected into normal female rats, 30 to 35 days old with a body weight from 30 to 45 g. The rats are killed on the fifth day, and the vagina is prepared for microscopical examination. Mucification is produced by a fraction of the amount of extract required for the production of vaginal cornification and uterine enlargement. The method allows of the determination of very small amounts of gonadotropin. Although the method is rather time-consuming it is of value especially for the assay of the small quantities of gonadotropin present in normal (non-castrate) urines.

4. Assay of Commercial Hypophyseal Gonadotropins

Commercial preparations of hypophyseal origin are few in comparison with those prepared from the urine of pregnant women and from the pregnant mares' serum. While the assay of the two last-mentioned groups of preparations does not present any serious problems because

their activity can be expressed in international units of the standard preparations, the assay of hypophyseal gonadotropins must be based upon the definition of various "animal units." The German preparation Preloban, Bayer, was assayed in "Reifungseinheiten" ("maturation units") on young cockerels. Four to 6 weeks old cockerels received injection of the hormone daily for 6 days and were killed 8 days after the first injection. One rat unit was defined as the amount of the preparation which produced a 50% increase in the average weight of the testes.

The American preparation Synapoidin (Parke, Davis and Co.) contains a mixture of sheep pituitary extract and chorionic gonadotropin. The preparation is assayed on normal immature female rats and the "synergic unit" is the amount of substance which produces a fivefold increase in the average ovarian weight. Squibb's Prospermin, from normal male urine, and Gamone, from menopausal urine, are measured in uterine rat units.

VII. Summary and Conclusions

According to the source of origin and their different biological effects the gonadotropic hormones are divided into three main groups:

1. *Hypophyseal gonadotropin*, produced by the chromophilic cells in the adenohypophysis. The hormone probably consists of three factors, a follicle-stimulating (or gametokinetic) hormone, a luteinizing (or interstitial cell-stimulating) hormone, and a luteotropic hormone (identical with prolactin). The follicle-stimulating hormone is present in comparatively large amounts in the urine from individuals deprived of their gonadal function. The interactions of the different components of hypophyseal extracts make the quantitative assay on intact animals rather inaccurate. The most reliable results are obtained when hypophysectomized animals are used as test animals, the morphological changes in the gonads or in the accessory genital organs being used as criteria of response.

2. *Human chorionic gonadotropin*, produced by the chorionic epithelium in primate trophoblast and placenta. The hormone stimulates the formation of corpora lutea and the interstitial testicular tissue and is excreted in large quantities in the urine of pregnant women. The exact assay is facilitated by the establishment of an international standard preparation. The most reliable methods of assay are those carried out on immature female rats using vaginal cornification or ovarian weight as criteria of response, and those based upon the corpus luteum formation in immature mice.

3. *Pregnant mares' serum gonadotropin*, produced by the "endometrial cups" of the pregnant mare. The hormone has the same biological

effects as hypophyseal gonadotropin but these effects are probably due to one hormone. The serum gonadotropin is not excreted with the urine (or only to a very slight extent). An international standard preparation has been established and the best method of assay is the ovarian weight test in immature rats.

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Adrenocorticotropin

BY FRANCIS S. GREENSPAN, C. H. LI, M. E. SIMPSON,
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CONTENTS

Methods for the bioassay of adrenocorticotropic hormone (ACTH) have utilized changes in the weight, morphology, or chemical constituents of the adrenal cortex of normal or hypophysectomized animals. In general, the tests involving the intact animal have been useful as qualitative procedures, but for satisfactory quantitative work, hypophysectomized animals are to be preferred. Some of the methods which have been proposed are listed in Table I, and will be discussed in turn.

TABLE I

Methods for the Bioassay of Adrenocorticotropic Hormone

A. In the Intact Animal
  1. Increase in adrenal weight in the 21-day-old rat
  2. Increase in adrenal weight in the 4-day-old rat
  3. Increase in adrenal weight in the 2-day-old chick
  4. Assays based upon the metabolic and hematologic effects of the adrenal steroids released following administration of ACTH
B. In the Hypophysectomized Animal
  1. Repair of the adrenal cortex of the unilaterally adrenalectomized rat
  2. Repair of both adrenals in the immature rat
  3. Maintenance of adrenal weight and morphology in the adult rat
  4. Depletion of ascorbic acid or cholesterol content of the adrenal of the rat.

I. THE BIOASSAY OF ACTH IN THE INTACT ANIMAL

Moon (1937) has suggested a bioassay procedure using 21-day-old normal male rats. The amount of ACTH necessary to cause an increase of 50% over the adrenal weight of controls when injected into 21-day-old male rats in 3 doses over a period of 3 days was defined as one normal rat unit. A total dose of 20 mg. of an impure preparation of ACTH was usually found to be equal to one unit. From the data presented, how-

ever, it is apparent that there is considerable variation in the adrenal weights of both the control and the treated groups so that large groups of animals would be necessary for reliable determinations. In addition, the data do not fall onto a rectilinear log-dose response curve (Moon and Hansen, 1940). Finally, because of the large number of substances which will cause adrenal hypertrophy in the intact animal (Tepperman *et al.*, 1943), it is clear that this reaction is not at all specific. Therefore, because of the insensitivity, lack of specificity and low degree of accuracy, this method has not been widely used.

The sensitivity of the test in the intact animal was increased somewhat by using 4-day-old rats (Moon, 1940). In this procedure, rats were grouped into litters of 8, both male and female, on the day of birth. Beginning the fourth day post-partum, the rats in each litter were injected intraperitoneally with 0.1 ml. of an ACTH preparation once daily for 3 days. The litter was sacrificed 24 hours later and the adrenals and thymuses dissected out and weighed. It was found that a 50% increase in the weight of the adrenals could be brought about with one-twentieth the amount of hormone necessary for a unit response in the 21-day-old normal rat test. On the other hand, the data presented do not fall onto a linear log-dose response curve, and it must be said that this modification increases the sensitivity, but not the accuracy or specificity of the test. In addition, the 4-day-old rat test has the disadvantage that impure extracts are toxic to the young animals.

Bates, Riddle, and Miller, in 1940, suggested the use of the 2-day-old chick for the bioassay of ACTH. White Leghorn cockerels, 2 days old, were injected subcutaneously 3 times daily for 5 days. A unit of ACTH activity was defined as that amount of hormone which when injected as above, in groups of 10 animals, produced an increase in adrenal weight of 25% above the controls. The assay data for a single partially purified ACTH preparation (No. 729) falls onto a linear dose-response curve, for which the following mathematical expression was obtained:

$$Y = 10.1 + 0.267X$$

where Y is the adrenal weight response in milligrams and X the total dose ACTH (impure material) in milligrams. Ten milligrams of this preparation was equal to 1 chick adrenal unit. The authors noted the variation in the control weights of the adrenals in chicks from different hatcheries and considered it advisable that adrenal weights in control animals be obtained with each run and the increases in treated animals be expressed as per cent increase over the control for that run. This procedure would seem to be the only one of those using the intact animal which might be

suitable for quantitative analysis; it is however relatively insensitive and lacks specificity.

Evidence of the potency of ACTH can be adduced from the metabolic or hematologic effects of the adrenal steroids released when ACTH is administered. The metabolic effects of ACTH have been summarized by Li and Evans (1947) and include inhibition of growth, effects upon nitrogen, carbohydrate and fat metabolism, effects upon lymphoid tissue and serum proteins, and effects upon steroid excretion. The response of the normal human adrenal to a given dose of ACTH has been studied by Hills *et al.* (1948) and Forsham *et al.* (1948), and a simple test procedure based upon the fall in circulating eosinophils and the rise in uric acid excretion following the administration of ACTH has been evolved (Thorn *et al.*, 1948). Mason *et al.* (1948), have studied changes in urinary steroids following the administration of ACTH. In a sense, this type of response is a qualitative index to the biological effectiveness of the ACTH administered, and it may be that these reactions will be helpful in ascertaining the biological potency of ACTH or its derivatives.

II. The Bioassay of ACTH in the Hypophysectomized Animal

The great advantage of using hypophysectomized animals for the bioassay of ACTH is that the source of the animal's own secretion of ACTH is removed and the effects noted are due entirely to the administered material. This immediately increases the sensitivity and specificity of the methods tremendously and makes reliable quantitative analysis possible.

Smith (1930) first demonstrated that atrophy of the adrenal cortex followed hypophysectomy in the rat, and that implants of anterior pituitary would prevent this atrophy. Collip *et al.* (1933, 1934) suggested the use of the unilaterally adrenalectomized, hypophysectomized rat for the bioassay of ACTH. Rats were hypophysectomized 11 to 148 days prior to assay. The left adrenal was removed 8–23 days posthypophysectomy and weighed and sectioned as a control. ACTH extracts were injected intraperitoneally twice daily for one week, and then the right adrenal was removed, weighed, and sectioned. The amount of ACTH necessary to bring about the "repair" of the right adrenal to a normal control level was considered to be one unit.

The bioassay of ACTH by repair of both adrenals of hypophysectomized immature rats has been standardized by Simpson *et al.* (1943). Female rats, 26 to 28 days of age, were hypophysectomized and the adrenals were allowed to regress for 14 days. At this time, daily intraperitoneal injections of ACTH were given for 4 days, followed by autopsy

24 hr. after the last injection. For microscopic sections, the adrenals were fixed in formol, cut as frozen sections, and stained with Sudan Orange. Following hypophysectomy, the adrenal weight is reduced from 18 to 9 mg. and marked changes in the distribution of lipids occur. The minimal response to the administration of ACTH was a redistribution of the lipid, even before changes in total adrenal weight occurred. This minimal response could be obtained with a total dose of 0.010 to 0.025 mg.
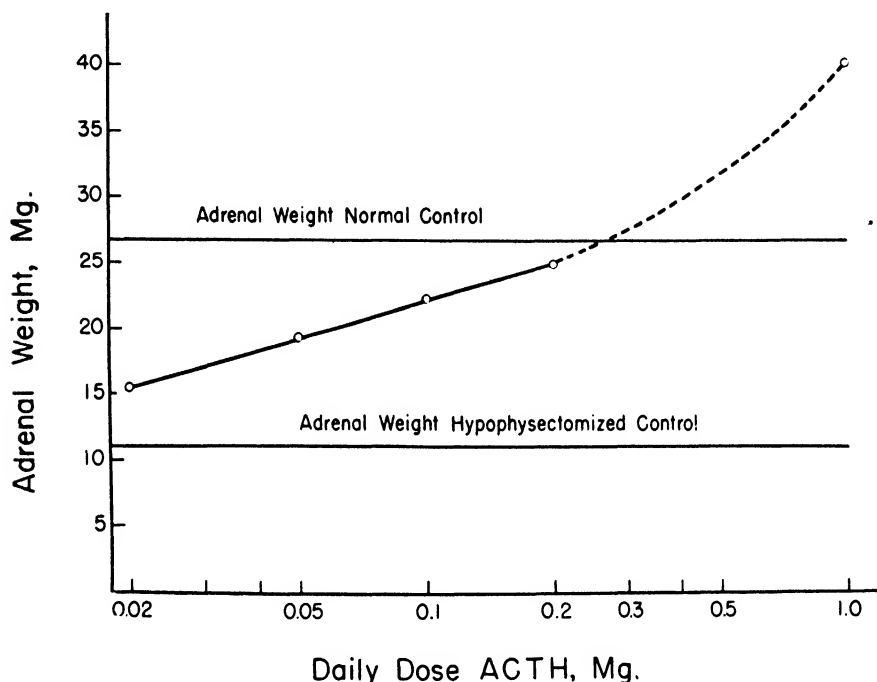
Fig. 1. The maintenance of adrenal weight in 40-day-old hypophysectomized male rats treated for 15 days with pure ACTH. (After Simpson, Evans, and Li, 1943.)

of pure ACTH (Li *et al.*, 1942). This test is both sensitive and specific; on the other hand, it has a low degree of accuracy. The response at different dose levels depends mostly upon histological interpretation, since the adrenal weight response is variable over the short injection period. Sayers *et al.* (1943) have used 45-day-old male rats, 2 weeks post-hypophysectomy, injected intraperitoneally 3 times daily for 3 days. Their results are essentially similar to the above.

The maintenance of normal adrenal morphology and weight by injection of ACTH immediately after hypophysectomy was suggested by Astwood and Tyslowitz (1942). As standardized by Simpson *et al.* (1943), the method is as follows. Male rats, 40 days old were used

because a wider range in adrenal weight loss was available in which the response to graded doses could be measured.   The animals were hypophysectomized and injected intraperitoneally daily (except Sunday) for 15 days (13 injections).   A unit was defined as the daily dose which maintained the preoperative adrenal weight under these conditions.   A daily dose of approximately 0.2 mg. of pure ACTH, or a total dose of 2.6 mg., was equal to 1 unit.   Between adrenal weights of 12 mg. (hypophysectomized 40-day-old rat) and 28 mg. (normal 40-day-old rat), increasing doses of ACTH produced increments in adrenal weight that fall into a linear log dose-response relationship.   (Table II, Fig. 1.)   Groups

TABLE II

Bioassay of Adrenocorticotropic Hormone in 40-Day-Old Hypophysectomized Male Rats (Maintenance Test)[a]

Daily dose[b] mg.	No. of rats	Average BW at autopsy g.	Adrenal weight average mg.
1.0	10	107.8	40.1 ± 0.8[c]
0.20	15	117.4	25.1 ± 0.9
0.10	15	117.9	22.3 ± 1.0
0.05	24	118.6	19.5 ± 1.0
0.02	14	107.8	15.6 ± 0.7
0.00	19	127.5	12.0 ± 0.5
Normal control 40 days	171	130.0	28.3 ± 0.41

[a] From Simpson, Evans, and Li (1943).
[b] A single injection daily.
[c] The $\pm$ values are the standard error.

of 15 to 25 animals are probably necessary, however, for satisfactory data.   Sayers *et al.* (1943) using a similar procedure on 45-day-old male Sprague-Dawley rats were unable to obtain such a linear relationship, but considered the method satisfactory in terms of the "unit" response. The maintenance test has, in general, been considered more satisfactory than the repair test (Simpson *et al.*, 1943), but it has the disadvantages that a 2-week injection period is necessary, a large total amount of hormone is required, and the accuracy of the determination at various dose levels is relatively poor.   It should be pointed out that in all of the preceding methods, increasing the frequency of injection greatly increases the response, presumably because of the rapidity of destruction of ACTH.   The number of injections daily must be standardized for each procedure.

By far the most sensitive and accurate method for the bioassay of ACTH has been the depletion of adrenal ascorbic acid, as standardized by Sayers *et al.* (1948).   In this method, male rats, weighing 120–160 g.,

were maintained at a constant temperature for 1 week prior to hypophysectomy. Twenty-one to 27 hr. after hypophysectomy, the animals are anesthetized with sodium pentobarbital intraperitoneally, and the left adrenal is removed. The solution to be assayed is injected via the tail vein and one hour later the right adrenal is removed. The adrenals are carefully freed of connective tissue under a dissecting lens, and weighed on an analytical balance. They are homogenized with sand and 4% trichloracetic acid in a glass homogenizer, and the total ascorbic acid content is determined by the method of Roe and Keuther (1943). It has been found that the ascorbic acid content of the two glands of the hypophysectomized animal does not differ by more than 25 mg./100 g. of tissue, and that the depletion of the ascorbic acid concentration of the remaining adrenal is a specific function of the dose of ACTH administered. A rectilinear relationship exists between the depletion and logarithm of the dose over the range of 0.15 to 2.5 μg. of highly purified ACTH. In groups of 12 or more animals at each dose level the method has been found to be extremely precise, having an average index of precision (λ) equal to 0.176 ± 0.016.

In our experience the Sayers method has been most satisfactory. The data obtained in the standardization of pure ACTH prepared according to the method of Li et al. (1943) are presented in Table III and Fig. 2. We have found that 0.2 μg. ACTH/100 g. body weight

TABLE III

The Depletion in Adrenal Ascorbic Acid in Hypophysectomized Rats Following Intravenous Administration of Pure ACTH

Dose ACTH μg./100 g. BW	Depletion ascorbic acid mg. %[a]	Significance of difference between means[b]	
		Groups compared	P value[c]
Controls	−6 ± 8.3 (27)		
0.2	−43.3 ± 7.3 (23)	0.2 vs controls	< 0.01
0.5	−59.9 ± 3.2 (10)	0.5 vs 0.2	0.25
1.0	−73.0 ± 6.4 (13)	1.0 vs 0.2	< 0.01
		1.0 vs 0.5	0.10
2.0	−100.5 ± 6.3 (11)	2.0 vs 1.0	< 0.01
3.0	−100.7 ± 11.6 (12)	3.0 vs 2.0	0.50
5.0	−115.8 ± 6.9 (13)	5.0 vs 3.0	0.25
		5.0 vs 1.0	< 0.01
10.0	−145.4 ± 4.8 (8)	10.0 vs 5.0	< 0.01
20.0	−149.3 ± 9.7 (10)		
50.0	−194.8 ± 6.9 (5)		

[a] Mean depletion in mg./100 g. fresh tissue ± standard error corrected for small groups. Figure in parentheses equals number of animals.
[b] Corrected for small groups.
[c] From "T" table, Fisher and Yates, 1938.

produced a significant depletion of the adrenal ascorbic acid. The most satisfactory working range of the curve lay between 0.2 and 10 μg. From calculations of the significance of the difference between the means at each dose level it can be shown that for groups of 10 to 12 animals, an increase in dose of approximately 5 times was necessary to produce a significant increment in the response. In our hands, the accuracy of the method has not been as high as that achieved by Sayers. From our data, the slope of the log-dose line $b = 56.6$, and the standard deviation about the line $S = 27.9$, whence λ equals 0.499, which is a relatively poor precision. On the other hand, we have found the method to be extremely
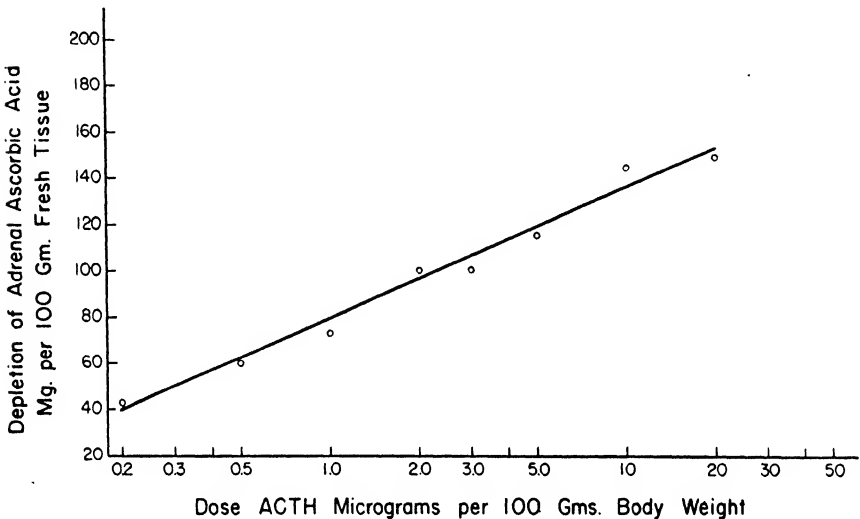
FIG. 2. Depletion of adrenal ascorbic acid following intravenous administration of ACTH.

sensitive, and very useful in the detection of small amounts of hormone in body fluids (Greenspan et al., 1950). We would re-emphasize the comments made by Sayers et al. (1948) regarding the necessity for rigorous standardization of the animals and the technical procedure and the importance of comparing an unknown to a standard run in parallel.

Munson et al. (1948) have modified the Sayers procedure in that both adrenals are removed simultaneously one hour after the intravenous administration of the hormone. Dehydroascorbic acid is determined by the method of Mindlin and Butler (1938). Sayers et al. (1948) have found that the same degree of accuracy may be obtained with this procedure provided that twice as many animals are used. We have found considerable variation in the response of the animals to this procedure in that a dose of hormone which produced a given response

one day might produce no response or a much greater response a few days later (Table IV). The slopes of the curves thus obtained were roughly parallel, but it has seemed that it would be necessary to run at least 2 or 3 standard points in groups of more than 12 animals as a control for each assay run, which would make this modification somewhat less satisfactory than the original Sayers procedure.

TABLE IV

The Variability of the Adrenal Ascorbic Acid Depletion Response in the Munson Modification[a]

Dose ACTH μg.	Adrenal ascorbic acid content[b] of both adrenals, mg. %[c]			
	Run I	II	III	IV
Control	493 ± 13.1 (5)	426 ± 17.8 (4)	470 ± 7.8 (4)	426 ± 20.9 (4)
0.5	377 ± 3.1 (5)		406 ± 30.1 (4)	346 ± 11.8 (4)[*]
1.0		451 ± 17.5 (3)		
5.0	326 ± 16.5 (4)	384 ± 29.7 (3)		296 ± 9.7 (4)
10.0			369 ± 17.0 (3)	
20.0				272 ± 23.2 (3)
50.0			310 ± 29.3 (4)	

[a] See text for details.
[b] Dehydroascorbic acid (Mindlin-Butler method).
[c] Mean ± standard error corrected for small groups. Figure in parentheses equals the number of animals per group.

The depletion of the cholesterol ester content of the adrenal following stress, or the administration of ACTH has been studied by Sayers *et al.* (1944, 1945, 1946), Levin (1945), and Ludewig and Chanutin (1946), and has been reviewed by Sayers and Sayers (1949). In summary, about 3 hr. after the injection of a single large dose of ACTH the cholesterol content of the adrenal has dropped to 50% of its normal value; in 12 hr., the cholesterol begins to re-accumulate, and in 24 hr. it has returned to its normal value. The changes are similar in degree to the changes in adrenal ascorbic acid, but occur at a slower rate. Ascorbic acid changes have been more thoroughly studied as a bioassay procedure principally because the method for the determination of ascorbic acid is simple and highly accurate.

It should be pointed out that the factor of ACTH which brings about the depletion of the adrenal ascorbic acid may not be the same as the factor necessary for the maintenance of the adrenal in the hypophysectomized animal. Certain observations by Li and Greenspan (1949) have suggested that several ACTH preparations which have had comparable activity by the maintenance test have not had comparable activity by the ascorbic acid depletion test. At the present time it

might be advisable to utilize both the ascorbic acid depletion test and a test based on adrenal morphology of the hypophysectomized rat.

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Thyrotropic Hormone

By C. W. TURNER

CONTENTS

I. INTRODUCTION

The development of our knowledge of the role of the anterior pituitary gland in the regulation of thyroid gland growth and secretory function is one of the most interesting phases of pituitary endocrinology. With a background of the past 20 years of research in this field, some of the earlier papers may be pointed to as indicating the role of the hypophysis in thyroid control, but the investigations of Smith (1927–30) upon the effects of hypophysectomy and replacement therapy on the thyroid gland of the rat and the observations of Loeb and Bassett (1929) and Aron (1929) upon the effects of bovine anterior pituitary extracts upon the thyroid glands of guinea pigs dramatized the importance of the pituitary in thyroid regulation and set in motion the intensive research of the past two decades.

Smith (1916) and Allen (1916–17) showed that following the ablation in the tadpole, in the late egg stage, of the ectodermal bud destined to form the hypophysis, there resulted a failure of the thyroid to undergo normal development. Allen (1920) reported that replacement therapy with frog pituitaries was effective. Smith and Smith (1922–23) demonstrated that the administration of the bovine anterior lobe, but not the other lobes, induced reparative effects in the thyroid. It was further shown that the feeding of fresh anterior lobe had no restorative effect.

Smith (1927a, b, 1930) noted involution of the thyroid of rats after hypophysectomy. Again, the feeding of fresh bovine anterior pituitary glands (2 or 3 daily) gave no reparative effects; moreover, saline suspensions of similar tissue also failed. When fresh rat pituitaries were implanted, however, pronounced reparative changes were produced. When the implants were stopped the glands again underwent atrophy.

Loeb and Bassett (1929) reported that the thyroid glands of guinea pigs which received injections of acid or alkaline extracts of cattle anterior pituitary gland showed within a short time a marked hypertrophy followed by a loss in body weight. It was soon shown by Silberberg (1929) that a distinct hypertrophy of the thyroid gland of this animal occurred as early as one day after injection. Siebert and Smith (1930) noted a very rapid marked rise in the basal metabolism, while Hageman and McCordock (1932) noted rises in the heart rate and nervous irritability following the administration of anterior pituitary extracts. Closs et al. (1932) showed that the injection of acid anterior pituitary extracts caused an increase in the organic blood iodine at the expense of the organic iodine content of the guinea pig thyroid gland.

The above observation of the thyroid stimulating action of the anterior pituitary by Leo Loeb and his associates in St. Louis was con-

firmed by Aron (1929–30) and many others later. In addition to the guinea pig, the effect upon cats, dogs, and rabbits was also studied. The responsiveness of the bird's thyroid to pituitary extracts was noted by Laraniov and Kotawa (1931), Laraniov *et al.* (1931), Riddle and Polhemus (1931) in doves and pigeons, and Schockaert (1931–32) in ducks. Thurston (1933) reported the responsiveness of various species to bovine pituitaries to be as follows: (1) mouse, (2) rabbit, (3) cat, (4) pigeon and (5) guinea pig, the mouse showing the least and the guinea pig the greatest effects of the injections.

1. Isolation

Since the early work of Loeb and Bassett (1930) in the extraction of thyrotropic hormone from bovine anterior pituitaries, progress in the isolation of this factor has been made by Janssen and Loeser (1932), Junkmann and Schoeller (1932), Anderson and Collip (1934), Rowland and Parkes (1934), Lambie and Trikojus (1937), Fraenkel-Conrat *et al.* (1940), Jorgensen and Wade (1941), White and Ciereszko (1941), Bergman and Turner (1942), White (1944), and Ciereszko (1945). The best preparations of bovine thyrotropic hormone from the Yale laboratory were reported by White (1944) to be homogeneous in both the Tiselius apparatus and the ultracentrifuge and had an approximate molecular weight of 10,000. One microgram of preparation contained 1 chick unit (Ciereszko, 1945).

2. Standard Preparation

The Third International Conference on the Standardization of Hormones agreed that extracts of the thyrotropic hormone should be assayed in comparison with an international standard preparation which was to be distributed by the National Institute for Medical Research, Hampstead, London, England. This standard was, however, never set up, although a preparation of whole ox anterior lobe was made available internationally to help in the comparative study of pituitary principles.

The conference agreed that only those assays can be considered safe which were based on actual observation of a stimulation of the thyroid, since other effects may be due to impurities in the extract.

Chance *et al.* (1939) stated that the (proposed) International Unit of thyrotropin was about equal to the guinea pig unit of thyrotropic activity described by Rowlands and Parkes (1934). Albert *et al.* (1946) reported that 15 mg. of the whole ox anterior lobe preparation contained 1 J-S unit as assayed by their combination chick method. Dvoskin (1948) compared a thyrotropin extract from hog pituitaries with the same preparation using the hypophysectomized rat in the intracellular droplet

method.   He found 3.5 μg. of the hog extract equal to 150 μg. of ox
pituitary preparation.

With preparations of thyrotropin of great biological potency now
available, there is a pressing need of an international standard preparation
which could be used in conjunction with the multitude of assay methods
which have been developed during recent years.

II. Factors Influencing the Sensitivity of the Thyroid Gland

1. Species

The great variation in the sensitivity of the thyroid glands of various
species to the administration of thyrotropic hormone was observed soon
after the role of the pituitary in thyroid regulation was discovered.   The
contrast between the guinea pig and the rat was first noted, then the
sensitivity of other species was determined.   The discovery of the
sensitivity of the domestic fowl's thyroid has been used to advantage
in the routine assay of this hormone.

It seems apparent that the variation in sensitivity is primarily related
to the rate of secretion of thyrotropic hormone.   Species such as the
rat and mouse have a relatively high concentration of thyrotropic hor-
mone in their pituitaries whereas the guinea pig and chick secrete rela-
tively little.

That strains of animals within a species may vary greatly in sensi-
tivity is indicated by the observations of Bates *et al.* (1941) in the case
of chicks.   These differences indicate the desirability of securing assay
animals as uniform genetically as possible.

2. Hypophysectomy

In species in which the pituitary secretes large amounts of thyro-
tropic hormone such as the rat, the removal of the hypophysis and the
endogenous source of hormone increases their sensit:vity to thyrotropin.
Only by this technic are such species made usable as assay animals.

3. Undernutrition

Stephens (1940) pointed out an alternative technic by which the
endogenous secretion of thyrotropic hormone could be reduced without
hypophysectomy.   Undernutrition in the guinea pig, of sufficient degree
to cause a loss of 20 to 30% of body weight in a period of 2 weeks, resulted
in atrophy and flattening of the acinar epithelium and retention of colloid,
suggesting a resting, inactive gland.   The thyroids of such animals
showed a marked increase in sensitivity to the thyrotropic hormone.

Injection of as little as one-tenth the minimal effective dose for normal animals may be observed in underfed animals.

4. Temperature

Since environmental temperature plays an important role in stimulating the secretion of the thyrotropic hormone, high temperatures should be expected to increase the sensitivity of the animal to thyrotropin and low temperatures lower the sensitivity (Baillif, 1937). In the case of chicks a relatively high temperature is maintained routinely; in other assay animals the maintenance of a uniform high environmental temperature should prove advantageous.

5. Administration of Thyroid Hormone

The injection of thyroxine or the feeding of desiccated thyroid or thyroprotein to animals is known to depress the secretion of the thyrotropic hormone [see Adams and Jensen (1944) for review]. It is the basis for the assay of the thyroid hormone using thiouracil and other goitrogenic chemicals. It has been suggested that more uniform results might be obtained in the assay of thyrotropin if the animals were pretreated with sufficient thyroprotein to depress the endogenous secretion of thyrotropin. However, the observations of Cortell and Rawson (1944) raises a question as to the feasibility of this plan since they concluded that the presence of circulating thyroxine definitely interferes with the response of the thyroid gland to injected thyrotropic hormone. In the use of normal animals, however, the elimination of the variation in the response due to endogenous thyrotropin secretion might offset the direct effect of the thyroxine.

III. METHODS OF ASSAY OF THYROTROPIC HORMONE

As was natural, the early methods of assay were rather crude and yielded only qualitative results. The improvement in methods has made possible not only more quantitative results but, by reduction in the amount of hormone required for the assay, has made it possible to assay the pituitaries of experimental animals and body fluids. It is interesting to note that the guinea pig which was used by Loeb in his early work is still the species of choice by many investigators. The second most popular species has been the chick. Its small size, sensitivity, and availability throughout the year are reasons advanced in its favor. The variability in response is one of the chief objections to its use.

In describing the assay methods it was decided to classify the methods on the basis of the animal of choice, starting with the guinea pig.

1. Guinea Pig

A. GRAVIMETRIC METHODS

The observation that marked increases in thyroid weight followed the administration of thyrotropin in guinea pigs was adopted by a number of investigators as a criterion of response (Loeb and Friedman, 1931). Rowlands and Parkes (1934) defined a unit as the amount of extract, which given daily for 5 days, will cause the thyroids of the immature guinea pig (150–300 g.) to attain a weight of 60 mg. (or double their weight). It was suggested that the thyroid weight bears a linear relationship to the logarithm of the dose, however, Emmens (1940) showed that a logistic curve was to be preferred. This curve, passing through the control thyroid gland weight of 30 mg., is asymptotic to a limit of approximately 90 mg. and has the equation

$$Y = \frac{90}{1 + e^{0.69 - x}}$$

Aron (1936) defined 100 guinea pig units as the amount, which, injected at one time into young guinea pigs weighing about 200 g., stimulates in 24 to 36 hr. a 50–100% increase in the weight of the thyroid accompanied by signs of hypersecretion. On this scale 10 units produced visible effects on the activity of the thyroid, while 40–50 units caused the cells to begin to multiply.

Reece and Turner (1937) defined a unit as that amount of hormone required to produce a 50% weight increase in the thyroids of 4 male guinea pigs weighing between 140–170 g. after 4 daily injections with weighing on the fifth day.

As a result of the extensive use of guinea pigs in the assay of thyrotropin by Bergman and Turner (1939), a guinea pig unit was defined as the total amount of hormone administered over a 5-day period, with subcutaneous injection once each day which will cause a mean weight increase of 50% (to about 26.4 ± 1.63 mg.) in the thyroids of 10 male guinea pigs weighing an average of 155 ± 15 g.

Although the response of increasing dosage is usually most sensitive in the region of 50% response, one is justified in calculating the unitage at somewhat lower or higher thyroid weights.

B. HISTOLOGICAL METHODS

The administration of thyrotropic hormone to immature animals with inactive thyroid glands is followed by colloid resorption, increased vascularity, and increased epithelial cell height. Junkmann and Schoeller (1932) defined a unit of thyrotropin as the amount required to produce a

definite histological reaction in one out of two guinea pigs weighing between 100 and 150 g. after three daily injections.

Heyl and Laqueur (1935) observed three levels of activity in the thyroid gland of the young guinea pig, an inner central zone in a continued state of flux, a middle zone which reacts readily to thyrotropin, and a peripheral zone which is comparatively inactive. It is suggested that changes in the middle zone be used as an index of activity.

The Junkmann and Schoeller method was modified by McGinty and McCullough (1936) to the use of 180- to 200-g. guinea pigs which were injected on 4 successive days. Thyroids were removed on the sixth day. Several regions of the thyroid are examined and rated +1 to +4. They reported that one-fourth of the controls show a +1, and one out of 20 a +2 rating. A unit effect is defined as a +2 reaction in 3 of 4 animals on a given dosage level and a confirmatory +3 response on the next higher dosage group.

Jorgensen and Wade (1941) defined a guinea pig unit as the minimum quantity of extract injected in 6 equal doses over 72 hr. required to effect histological changes involving an increased height of the epithelium and a definite decrease in stainable colloid around the periphery. They state that this unit is essentially the Junkmann and Schoeller unit although 75- to 150-g. animals were used.

That the adrenal gland plays no role in the response of the guinea pig thyroid to thyrotropin was shown by Robinson and Trikojus (1947) who observed that the adrenalectomized animal responds similarly to the normal animal both in weight and histological change.

C. MITOTIC INDEX

Changes in the mitotic activity of the epithelial cells of the guinea pig thyroid were used as a measure of thyrotropic hormone activity by Kippen and Loeb (1935). The thyroids were fixed in Petrunkevitch solution and embedded. One lobe was cut into complete serial sections, each section being approximately 6 μ thick. The mitoses were counted in every tenth or twentieth section. The average number of mitoses in a section was multiplied by the number of sections into which the thyroid was cut and thus the total number of mitoses was obtained.

Following the first injection only a slight rise in proliferative activity was observed after 24 hr. with all doses. A maximum was reached after two injections. In groups on low levels, a sudden and steep decline then takes place. On higher levels of hormone the activity remains high for several days, but then a rapid decline takes place. It was concluded that a small dose can transmit to the acinus cells a proliferative momentum about as great as larger doses, but that cells thus stimulated

are not able to maintain the level reached as well as those stimulated with the larger doses.

Bastenie and Zylberszac (1937) suggested the use of 0.025 mg. of colchicine for every 30 g. of body weight to 220- to 250-g. guinea pigs to arrest the mitotic division in the metaphase. The drug should be given about 9 hr. before killing the animals.

D. MICROHISTOMETRIC METHODS

Starr *et al.* (1936–39) and Rawson and Starr (1938) described a micro-histometric method of assay for thyrotropic hormone using guinea pigs. The assay animals weighing from 180 to 225 g. were injected with the extract daily for 3 days, autopsied on the fourth day, and the thyroids were fixed in formalin. The mean cell height of control animals was 3.77 μ. Graded doses of thyrotropic hormone caused increases to 6.14 μ. Extracts of urine in some cases indicated the presence of thyrotropin.

Adams and Allen (1942) used a modification of this method along with several other indices to measure the thyrotropin of the mouse pituitary.

Later Starr and Metcoff (1941) reported that a single subcutaneous injection of thyrotropic hormone will result in a significant increase in mean acinar cell height within 8 to 16 hr., which is comparable to that heretofore reported in 72 hr. following a series of daily injections. This response persists at least 48 hr. following injection.

E. INTRACELLULAR COLLOID DROPLET METHODS

An assay method based on the determination of the number of colloid droplets found in cells of the guinea pig thyroid prepared by freezing-drying was described by De Robertis and Del Conte (1944). This cytological method is claimed to be several hundred times as sensitive as previous methods. It is capable of detecting the injection of 0.0002 Junkmann-Schoeller unit.

De Robertis (1948) describes the use of this method as follows. Guinea pigs weighing 150–300 g. are lightly anesthetized with ether, and the extract is injected into the heart. The animals are sacrificed by a blow on the head 30 min. after the injection, and the thyroids are dissected out as quickly as possible and frozen in isopentane at −195°C. or in liquid air. The best cytological results are obtained by putting the gland between two pieces of muscle prior to freezing. The glands are then dried *in vacuo* at −30°C. in Gersh's apparatus, treated in absolute alcohol to denature the proteins, and embedded in nitrocellulose. Sections are stained with aniline blue-orange G by the method of De Robertis (1941). The number of droplets in 10 follicles of the inner zone of the gland are counted and the total number divided by the sum

of the average diameters of the follicles. This quotient multiplied by 100 represents the number of droplets contained in a hypothetical follicle 100 microns in diameter, and is called the cytological coefficient (C_c).

It is claimed that this is the first method which is sufficiently sensitive to detect definitely and to measure the level of circulating thyrotropic hormone in normal blood. The success of the procedure depends on separation and extraction according to Fellinger's (1936) method, which removes the fraction of the blood containing the thyroid hormone so that almost no iodine is left and permits the recovery of thyrotropin after it has been added to the blood.

Junqueira (1947) has suggested a modification of the intracellular colloid droplet method using an *in vitro* technic. The thyroid gland is removed and cut into small fragments weighing about 1 mg. each. These are placed in Carrel flasks in suitable nutritive media. The thyrotropic hormone or other test material is added, and after a suitable time the tissue is prepared for examination by the method described above. It was shown that the thyrotropic hormone acts directly on the thyroid cells and proved that neither innervation nor vascularization are necessary for this action.

Sodium iodide in a concentration of 0.15 M to 0.015 M inhibited the action of thyrotropin on the secretion and release of the colloid.

F. IODINE CONTENT OF THYROID

The reduction in the iodine content of the guinea pig and chick thyroid following the administration of the thyrotropic hormone was suggested by Stimmel *et al.* (1936) as a suitable criterion of response as an assay method. They defined a unit as the smallest amount of substance, which, following intraperitoneal injection on three successive days, caused a 50% decrease in the percentage of iodine in the thyroid glands of normal guinea pigs (25.5 μg. % to 12.7 μg. %) whose body weight is not more than 275 g. and not less than 225 g., when an average of at least four animals was used.

Cuyler *et al.* (1936) prepared a preparation which was observed to contain 1 unit per 0.75 mg. In tests with 21-day-old rats three doses of 4 mg. of this preparation were found to decrease only slightly their iodine content. Three doses of 0.025 mg. were found sufficient to accelerate the rate of metamorphosis in the frog tadpole.

2. Chick

A. GRAVIMETRIC METHODS

Smelser (1937–38) used the day-old chick in the assay of thyrotropic hormone. For the assay 5 chicks were injected daily subcutaneously for 5 days with autopsy 24 hr. after the last injection. The control chicks

had thyroids weighing an average of 2.8 mg., whereas dosages of 0.1 to 20.0 mg. of beef anterior pituitary extract stimulated thyroid weights of from 3.9 mg. to 20.3 mg. Chicks receiving smaller amounts of extract showed histological evidence of its activity.

Cope (1938) Kabac and Liapin (1938), Bergman and Turner (1939) and Fraenkel-Conrat et al. (1940) confirmed the above observations on the suitability of the day-old chick in the assay of the thyrotropic hormone. Cope reported that the chick thyroid could be doubled in weight by one-third of a Rowlands and Parkes guinea pig unit. Kabac and Liapin define a unit of thyrotropic hormone as the amount which brings the weight of both thyroids of the chick to 8 mg. (on the average) after daily subcutaneous injections during 5 days with autopsy on the sixth day. The use of a straight line obtained by relating thyroid weight and the logarithm of the dose (plotting on semilog paper) was suggested as a convenient method of assay of samples.

Bergman and Turner (1939) suggested a number of refinements in the assay of thyrotropic hormone using chicks. First, it was observed that there was a distinct difference in the average thyroid weight of male and female White Leghorn chicks examined on the sixth day (3.39 mg. for males; 4.82 mg. for the females). Further, for a given amount of thyrotropic hormone, the male thyroid weight increases almost twice as much as the female. From their data it appeared that a 50% increase in thyroid weight would be significant and yet would be in the most sensitive range.

A male chick unit of thyrotropic hormone was defined as the total amount of hormone administered over a 4-day period, with subcutaneous injections once each day, which will cause a mean weight increase of 50% (to about 5.4 ± 0.26 mg.) in the thyroids of 20 chicks weighing an average of 55 ± 10 g.

Fraenkel-Conrat et al. (1940) defined their chick unit as the total dosage which in 6 days causes a 33% increase in thyroid weights over that of the controls. This in chicks composed of both sexes corresponds to the Bergman and Turner units of 50% increase in male and 20% increase in female chicks. Injections are subcutaneous, 0.2 ml. daily, for 5 days, with autopsies 24 hours after the last injection. The curves obtained when thyroid weight increase (up to 200%) is plotted against the logarithm of the dose are generally straight lines. One unit causes a 33% increase, 2 units 92%, 3 units 125%, etc.

Bates et al. (1941) observed two strains of White Leghorn chicks which differed greatly in their responsiveness to thyrotropic hormone. Chicks from one source required 4 times as much thyrotropin to produce an equal amount of stimulation as measured by an increase of thyroid

weight as did chicks from the other source; and maximum stimulation during a 5-day test yielded thyroids weighing only 15 mg. in one type of chick and 35 mg. in the other more sensitive type.

B. HISTOLOGICAL METHODS

In order to assay the thyrotropic hormone in urine extracts, Jones (1939) developed a method using chicks but the response was based upon histological examination of the thyroid glands after being fixed in 10% formalin. The normal chick gland showed acini lined by flat epithelial cells which contained a flat or oval nucleus and but little cytoplasm. In 2 of 46 untreated chicks, the cytoplasm was fairly abundant and the nucleus was rounded. When the two diameters of many of the cells were equal or when the epithelium become frankly columnar, then this was taken as an indication of increased thyroid activity.

The thyroid epithelium was considered to be definitely stimulated (+) when the majority of the acini were lined with columnar or cuboidal epithelium, (±) when some of the cells appeared to be slightly stimulated and approached cuboidal epithelium, and (−) when the epithelium lining the glands was flat and ribbon-like.

Jensen and Grattan (1939) suggested the following chick unit of thyrotropic hormone based on the histological examination of the thyroid: "A chick unit is the total amount of hormone which, when injected subcutaneously into 4-day-old chicks, twice daily for 4 days, will show minimal stimulation of the thyroid gland by the 5th day after the onset of injection."

Jorgensen and Wade (1941) defined a chick unit as the minimum quantity of extract injected in 4 equal doses over 48 hr., necessary to effect histological changes in the thyroid involving increase in cell size and staining reaction rather than resorption of the colloid.

Ciereszko (1945) preferred a 3-day-old White Leghorn chick, injected for 5 days with a volume of 0.5 ml. each day and sacrificed 24 hr. after the last injection. The minimum amount of extract required to produce a definite positive histological response in eight chicks was considered one unit. He was not able to obtain a significant correlation between gland weight and histological response.

C. MICROHISTOMETRIC METHODS

Rawson and Salter (1940) described a method of assay of thyrotropic hormone using day-old chicks of both sexes weighing 35 to 40 g. The birds were kept at 95°F. and fed a chick starter mash containing 0.1% iodine. The chicks are injected daily for 5 days with thyrotropic hormone. On the sixth day the thyroids are removed and fixed in 10%

formalin for about 2 hr. Paraffin sections were made and stained with hematoxylin and eosin. [Dvoskin (1947) suggested that fixation in Carnoy's fluid would cause less shrinkage in cell height.]

Under the oil immersion lens, the sections are examined until 100 successive, clearly delineated acini have been observed. From each of these acini a representative cell is chosen and its height measured with a Leitz echelon micrometer. No cell should be measured unless its limits are clearly visible. From these measurements the mean and standard deviation is determined. The effect of increasing dosage of thyrotropic hormone upon mean cell height is shown in Table I.

TABLE I

Response of Acinar Cell Height to Increasing Amounts of Thyrotropic Hormone

Daily dose			No. of chicks	Average mean cell height (μ)	Average standard deviation
Controls			8	2.46	0.45
⅛ Junkmann-Schoeller U			8	3.03	0.49
¼	"	" "	10	3.62	0.52
½	"	" "	8	3.88	0.70
1	"	" "	9	4.18	0.53

It is suggested that this method is sufficiently sensitive to enable one to detect thyrotropic hormone in normal human urine.

Adams and Beeman (1942) modified the above method by determining the heights of the highest and the lowest cells in 50 follicles in a middle section of each gland at 1000 diameters with a Leitz ocular micrometer. They noted increases in thyroid cell height when examined 24 hr. after the implantation of a single pituitary of an adult male mouse. A unit was defined as that amount of fresh substance which will give a 100% increase in cell height of 5 to 10 1-day-old chicks 24 hr. after subcutaneous administration in a single dose.

D. INTRACELLULAR COLLOID DROPLET METHODS

The observations of a number of investigators that the administration of the thyrotropic hormone soon led to the formation of intracellular colloid droplets in the thyroid epithelium led Dvoskin (1947) to develop an assay method based upon this phenomenon in the chick.

Three-day-old White Leghorn chicks are injected with the thyrotropic hormone preparation. Two hours after the injection the thyroids are removed, weighed, and fixed in Carnoy's fluid for 1 hr. at room tem-

perature and rapidly embedded in paraffin.   Sections at 4 μ were stained by De Robertis' (1941) modification of Heidenhains' Azan stain.   Sections were allowed to remain in the phosphotungstic acid solution for only 1 hr.

Cell height of the thyroid epithelium of each animal was determined by averaging the height of a cell in each of 25 successive cross sections of follicles.   The number of intracellular colloid droplets of the thyroid epithelium of each chick was determined by totaling the number of large clearly visible droplets in the entire section thickness of 25 successive cross sections of follicles in a section through the midportion of the gland.   These measurements were facilitated by an orange filter.   The mean and standard deviation of the weights, cell heights and colloid droplets of the thyroid of groups of 20 chicks and P values comparing the means of different groups were calculated.

The sensitivity of the method is indicated by the following comparison. A chick weight unit as defined by Bergman and Turner required about 760 μg. of their thyrotropic preparation.   A Junkmann-Schoeller unit would equal about 640 μg.   The cell height of the thyroid epithelium increased above normal 2 hr. after a single injection in birds treated with 50 μg. or 100 μg. of pituitary extract.   As little as 10 μg. of hormone significantly increased the average number of droplets, resulting in more than a fourfold increase in their number.

De Robertis (1948) is of the opinion that the chick is not as satisfactory an assay animal as the guinea pig.   Whereas in the chick the minimum amount of hormone which can be detected is about 0.005 J-S unit, in the guinea pig 0.002 J-S unit may be detected.   This difference may be due in part to the greater variability of response in the chick, the small size of the colloid droplets, and the injection of the extract directly into the blood instead of subcutaneously.

Dvoskin (1948) reported that the hypophysectomized rat is more sensitive to thyrotropic hormone than the chick, and has the advantage of not having any colloid droplets in the thyroid epithelium as early as one day after operation.   However, he has induced droplet formation in the thyroid epithelium by a number of non-hormone agents.

E. THYROID IODINE CONTENT

Fraenkel-Conrat *et al.* (1940) determined the effect of thyrotropin upon the thyroid iodine of the chick under the conditions of the gravimetric method.   They observed that less hormone is required to cause a 50% drop in iodine content than to cause a 33% gain in thyroid weight. This test may serve to differentiate between beginning stimulation and biological variation around the control value.

F. COMBINATION METHOD

Albert *et al.* (1946) described an assay method based upon a combination of three methods. Ten to 20-day-old White Leghorn cockerels were injected once daily with a 1-ml. volume of extract subcutaneously for 3 days, and killed on the fourth day. The thyroid glands were dissected and weighed *en masse* on a torsion balance. After weighing, the
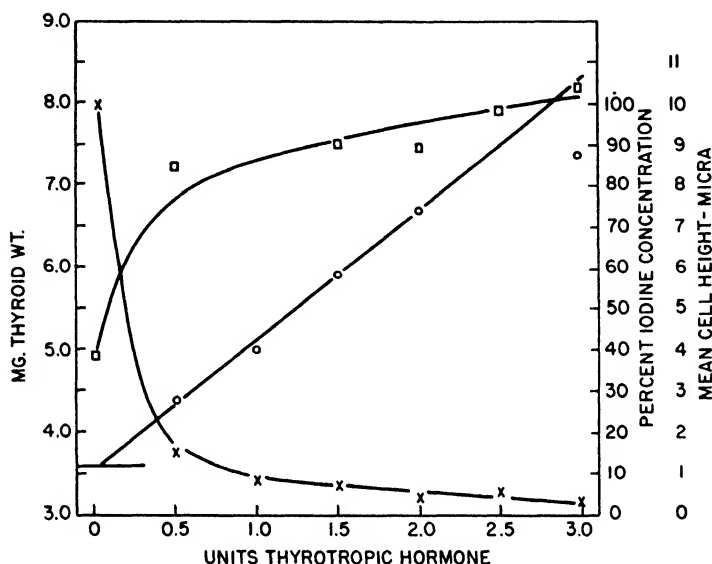
FIG. 1. The assay of thyrotropic hormone as based on the relationship between dosage and thyroid weight, thyroid iodine concentration, and mean cell height. Each point on the curves for thyroid weight and iodine concentration represents ten animals. Each mean cell height value is an average of 50 measurements on each of the five thyroid lobes of five different animals at any given dosage. □ values of mean cell height, ○ average thyroid weights, X iodine concentration values. The horizontal line at the lower left represents the range of thyroid weights of untreated chicks, 4 days of age. (From Albert *et al.*, 1946.)

glands were placed in 10% KOH and iodine determined. Finally, some of the thyroids were sectioned and the mean acinar cell height determined by the method of Rawson and Salter (1940). The results are shown in Fig. 1.

In practice the unitage is determined by reading the thyroid weight curve, but crucial experiments were checked by means of the cell height curve. Iodine concentration is used to indicate the positivity of the value obtained. When iodine was not given concurrently with thyrotropin, hyperplasia and hypertrophy of the thyroid do not occur without

an intensive and concomitant or previous fall in iodine concentration. With exogenous iodine, the fall in concentration is less.

G. COMPARISON OF GUINEA PIGS AND CHICKS

Smelser (1938) stated that the chick thyroid would react to one-tenth the amount of thyrotropic hormone required to affect the weight of the guinea pig thyroid. Cope (1938) reported that the dose of thyrotropic hormone per gram body weight necessary to double the weight of the thyroid gland was approximately the same in the chick as in the guinea pig, but due to the difference in body weight only one-third of the quantity is required for the former that is necessary for the latter. Kabac and Liapin (1938) stated that preliminary experiments showed that the chick was approximately 4 times more sensitive than the guinea pig.

The Bergman-Turner male chick unit of thyrotropic hormone was found to be about one-fourth the amount of their guinea pig unit. However, since the guinea pigs weigh about 3 times as much as the chicks, on an equal body weight basis, the male chicks are only slightly more sensitive then the guinea pig.

Fraenkel-Conrat *et al.* (1940) compared several assay methods using a thyrotropin preparation in which 0.011 mg. equalled 1 chick unit (equal to the Bergman-Turner chick unit). In 150-g. guinea pigs, histologically detectable thyroid stimulation of 7 out of 10 animals was obtained with a total of 4 c.u. administered for 3 days subcutaneously according to the J-S method. The Rowlands-Parkes guinea pig units required 17 c.u. It was observed that 2–4 c.u. were required to cause histologically detectable stimulation in 31- to 33-day-old pigeons injected subcutaneously or intramuscularly for 4 days. In immature hypophysectomized female rats, a histologically detectable response following either subcutaneous or intraperitoneal injections for 3 days was obtained with a total of 9 c.u.

3. Hypophysectomized Rats

A. HISTOLOGICAL METHOD

The hypophysectomized albino male rat was used by Hertz and Oastler (1936) for the assay of thyrotropic hormone in body fluids. Five days after the operation, injections were begun and continued for 5 days. The thyroids were then removed for histological section. Whereas the thyroids of the hypophysectomized rat shows marked decreases in cell height and large accumulations of colloid, the thyrotropic hormone caused maintenance of the height and mild vacuolization of the colloid.

B. INCREASE OF BASAL METABOLIC RATE

Anderson and Collip (1933–34) reported rises in the metabolic rate of guinea pigs and rats following the administration of thyrotropic hormone. They defined a unit as the smallest amount of hormone, which, when given twice daily to a hypophysectomized rat, will produce a 20% increase in oxygen consumption within 96 hr.

C. INTRACELLULAR COLLOID DROPLET METHOD

De Robertis (1942) noted that although the rat is less sensitive than the guinea pig to thyrotropic hormone, following the intraperitoneal injection of 0.5 or 1.0 guinea pig unit, colloid droplets appear in maximum number in the cells of the thyroid epithelium in 60 min.

Dvoskin (1948) made an extensive study of the usefulness of young (50–80 days) male hypophysectomized rats in the assay of thyrotropin . by the colloid droplet method. The thyroid epithelium of such animals contained no droplets one day after operation. Intracardiac injections of a hog pituitary extract was found to produce droplets in 30 min. with a maximal number at 60 min. While 1 μg. failed to show a response, 5 to 100 μg. showed increasing numbers of droplets. The difference in cell height between animals treated with 100 μg. of the hog extract and that of untreated controls was at the borderline of statistical significance, while 50 μg. treated animals was not significantly increased. Since 3.5 μg. of the hog extract were found to be equal to 150 μg. of I.S. preparation and this amount of extract gave borderline stimulation, it was calculated that this method could detect about 0.01 Junkmann-Schoeller unit.

Unfortunately, this assay method was shown to lack specificity to thyrotropin, since injections of typhoid vaccine, histamine, pilocarpine, and toxic human sera in sufficient amounts elicited droplet formation in all animals. While the method should be valuable in the assay of pituitary extracts, the assay of the thyrotropin content of blood and other body fluids would be open to question.

4. Tadpoles

Smith and Smith (1922) observed that the intraperitoneal injection of fresh anterior lobe substance of bovine hypophysis caused stimulation of the thyroid gland and metamorphosis in hypophysectomized tadpoles. Spaul (1924) reported that normal frog tadpoles could be similarly stimulated and Atwell (1934) caused marked acceleration of metamorphosis by the injection of thyrotropic extracts into hypophysectomized tadpoles.

Cuyler *et al.* (1936) reported that the emergence of the left front leg

of *Rana pipiens* tadpole was a sensitive indication of thyrotropic hormone. A positive reaction was obtained in 6 days with 0.025 mg. of extract administered every other day. One guinea pig unit was equal to 0.75 mg. of this same preparation.

D'Angelo *et al.* (1941) observed that total starvation of the frog tadpole prior to a critical developmental stage resulted in thyroid atrophy and a consequent metamorphic stasis which could be overcome with relatively small amounts of pituitary substance. An assay method based upon these observations was later developed (D'Angelo *et al.*, 1942). They defined a tadpole unit of thyrotropic activity as the minimal amount of hormone which, when given to the totally starved non-metamorphosing animal in 5 intraperitoneal injections on alternate days, will stimulate the thyroid gland, increase the hind limb length, and cause a body weight loss of 50% greater than in water-injected controls.

Direct comparison with the immature guinea pig (200 gm. body weight) indicates that the tadpole will respond to approximately $\frac{1}{60}$ to $\frac{1}{130}$ the amount of hormone which can be detected histologically in the guinea pig. These authors suggest that the sensitivity of the chick, guinea pig, and tadpole to thyrotropic hormone is of approximately the same order if body weight is taken into consideration.

Spaul (1924) reported success with a number of pituitary preparations in causing metamorphosis in axolotls, however, the time and the amount of hormone required for this purpose would exclude their use as assay animals.

5. Miscellaneous Species

A. DOVE OR PIGEON

Riddle (1931) suggested the use of the immature dove or pigeon in the assay of thyrotropin. He observed an increase of 79% in thyroid weight of 15 doves injected with extracts for periods varying from 3 to 11 days. Bates *et al.* (1941) reported that the average thyroid weight of White Carneau pigeons from the same source varied widely over a 5-year period. During the first 2 years the average weight was uniformly close to 40 mg. A stepwise increase in thyroid weight occurred in the autumns of 1938, 1939, and 1940, and in 1941 an average weight of 179 mg. was attained. Such marked changes in thyroid weight indicate the need of parallel tests with a standard preparation.

B. SPARROWS

Sparrows (*Passer domesticus*) were suggested as suitable assay animals in place of chicks by Vunder and Vibe (1940). They administered

pituitary extracts twice daily for 6 days. The thyroids were weighed the next day and sectioned. Increasing dosage led to increasing thyroid weight. No difference was noted in the sensitivity of the two sexes. Miller (1938) earlier noted that the thyroids of the English sparrow remained enlarged and hyperplastic following the injection of thyrotropic hormone over a period up to 5 months.

C. SNAKE

The grass snake was suggested by Mason (1938) as a suitable animal for the assay of the thyrotropic hormone. One-half unit of hormone administered twice at 24-hr. intervals stimulated hyperplasia and colloid loss when the thyroids were histologically examined on the third day. The thyroids were more responsive to the hormone when the snakes were maintained at 24°C. rather than at 13°C.   .

D. GOLDFISH

Two-inch goldfish were shown to be responsive to thyrotropin by Gorbman (1940). Groups of 5 fish, kept in 1-gallon jars, were injected intraperitoneally on 5 successive days with 0.2 ml. of extract in Ringer's solution each day. At autopsy on the sixth day, the lower jaw is removed and trimmed, fixed in Bouin's fluid containing acid to decalcify. The jaw is cut sagittally in 10 μ sections. The height of the thyroid epithelium was measured with a calibrated ocular micrometer. The average control epithelial cell measured 1 μ in height. A 100% increase in average follicle cell height (cells 2 μ high) was considered the minimal reaction.

ADDENDA

Based upon the earlier observation of Borell (1945) indicating a relation between thyroid cell height and the phosphate content of the thyroid gland, Borell and Holmgren (1949) suggest an assay method for the thyrotropic hormone based upon the increase in the uptake of radioactive phosphorus by the thyroid gland. After the thyroid is stimulated with thyrotropin, the number of labelled phosphate atoms in the thyroid multiplies nearly tenfold, while the cell height doubles.

Male guinea pigs weighing about 150 g. and maintained under a constant temperature of 30-32°C. are given injections of thyrotropin for two days. Radioactive phosphorus P^{32} in a dose of 0.05 mc. is injected intraperitoneally 40 min. before they are killed. The thyroid glands are removed and the radioactivity determined. It should be possible to adapt this new principle to the various types of assays and assay animals already described.

D'Angelo and Gordon (1950) reaffirmed their claims as to the value

of the stasis tadpole for the detection of minute amounts of thyrotropin such as is present in the blood serum and further described a method for the simultaneous detection of thyroxine. The International Gland Preparation of desiccated ox anterior pituitary used as a standard was said to cause approximately 50% increase in the thyroid weight of immature female guinea pigs (225 g.) following five daily doses of 10 mg. each. Seven and a half micrograms (0.0005 J-S units) or more of the preparation caused significant increases in the thyroid cell height and hind limb length. Thyroid activation and limb extension parallel one another, with both responses a linear function of administered thyrotropin. The stasis tadpole is also highly sensitive to thyroxine. As little as 0.025 μg. of D,L-thyroxine will reinitiate metamorphosis without causing significant change in the thyroid gland. In blood serum one can thus detect small amounts of thyroxine and thyrotropin simultaneously, or either separately.

The reader's attention is called to the review of Albert (1949) on the biochemistry of the thyrotropic hormone in which is presented a discussion of assay methods and a plea for an international standard thyrotropin so that the relationship of the various proposed assay methods may be determined.

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Lactogenic Hormone

By JOSEPH MEITES and C. W. TURNER

CONTENTS

I. Introduction

The pioneer work of Stricker and Grueter (1928, 1929) in France first demonstrated the presence of a lactogenic factor in the anterior pituitary. They initiated milk secretion in the mammary glands of pseudopregnant rabbits, which possess a well-developed mammary system readily responsive to lactogenic hormone. This work was soon

purified lactogenic hormone is unable to initiate lactation and (2) in intact lactating animals, whole pituitary extracts are more potent in increasing milk flow than purified lactogenic hormone. All this has an obvious bearing on the assay methods used to express lactogenic potency, and a considerable but unresolved discussion of this subject has already appeared in the literature (see Folley and Malpress, 1948, for latest review).

It is recognized that milk secretion is a complex phenomenon involving many factors other than lactogenic hormone. The precursors of milk come from the blood, which in turn come from the nutrition of the animal. Any condition which would interfere with the passage of milk precursors from the blood into mammary secreting cells, such as would occur during hypophysectomy, could be expected to interfere with milk production. Obviously the lactogenic hormone cannot stimulate mammary cells to secrete milk if some of the basic blood elements essential to the synthesis of milk are absent. The fact that whole pituitary extracts are more effective than lactogenic hormone alone in augmenting established lactation, may merely mean that products necessary for the synthesis of milk are made available to the mammary glands in greater abundance. It does not disprove the specificity of the lactogenic hormone.

Conclusive evidence of the direct action of lactogenic hormone on mammary tissue was first demonstrated by Lyons (1942), who injected the hormone directly into the milk ducts of rabbits and obtained a localized lactation under the site of injection. Later, by the use of the same technic, the present authors (1947) showed that *pituitary hormones other than lactogen, as well as thyroxine and adrenal cortical extracts, could not initiate lactation in pseudopregnant rabbits. Only the lactogenic hormone was specific in this respect.* Riddle and Bates (1939) had previously shown that only the lactogenic hormone was capable of initiating crop gland proliferation in pigeons. The burden of proof, therefore, remains on those who challenge the specific action of the lactogenic hormone on mammary tissue and its identity with the pigeon crop proliferating factor.

II. Mammalian Assay Methods

1. Introduction

Only the rabbit and guinea pig have proved satisfactory for mammalian assays of lactogenic hormone. The mouse and rat appear to be somewhat refractory to lactogenic hormone. There are several disadvantages inherent in mammalian methods of assay: (1) There is

no assurance that the amount of mammary gland growth will be the same in two animals, regardless of treatment. (2) It is difficult to judge quantitatively the amount of milk present in mammary glands, and no satisfactory methods have yet been developed for its measurement in small laboratory animals. (3) If the lactogenic preparation is not highly purified, other pituitary hormones present may augment the response above that obtained with lactogen alone. (4) Mammalian methods are not sufficiently sensitive to assay the small amounts of lactogen ordinarily present in the pituitaries of small laboratory animals, although Lyons' (1942) local intraductal technic in rabbits may make this possible. Against the above disadvantages there is the advantage that lactation constitutes a characteristic mammalian reaction to lactogenic hormone stimulation.

2. Rabbit Assay Methods

A. GARDNER AND TURNER (1933) METHOD

Gardner and Turner (1933) proposed the use of adult female rabbits whose mammary glands were developed to the condition of midpregnancy by induction of pseudopregnancy. The assay is conducted as follows:

i. Pseudopregnancy is induced in mature estrous females either by coitus with a vasectomized buck, or by the intravenous administration of 50 to 100 rat units of human chorionic gonadotropic hormone.

ii. The mammary glands are observed 12 to 14 days later to determine whether typical pseudopregnant growth has occurred. If pseudopregnancy has been induced, the gland is sufficiently developed so that it can usually be seen without difficulty by shaving a region of the abdomen or by making a small incision in the skin. Since the mammary glands are fairly thick at this time they can also be felt by manipulating the glands beneath the skin between the fingers.

iii. On the fourteenth to sixteenth day of pseudopregnancy daily subcutaneous injections of the lactogenic hormone are begun and continued for a period of 7 days.

iv. On the eighth day the rabbit is killed and the abdominal skin is incised in the midline and separated from the mammary glands underneath.

v. The mammary glands are rated for lactational response as follows:

Negative (−): absence of response.
Plus one (+): all ducts are filled with milk, lobules are flesh-colored and not enlarged.
Plus two (++): all ducts and most of lobules are filled with milk though not greatly thickened.

Plus three $(+++)$: entire gland is filled with milk.

Plus four $(++++)$: mammary glands are greatly extended with milk throughout.

vi. The rabbit unit is defined "as that minimum amount of extract which injected during a period of 7 days at daily intervals is required to induce a *plus three* $(+++)$ or *plus four* $(++++)$ response in rabbits previously pseudopregnant for a period of from 12 to 16 days."

B. BERGMAN, MEITES, AND TURNER (1940) MODIFICATION

Bergman *et al.* (1940) standardized the original Gardner-Turner method as follows:

i. Pseudopregnancy is induced in 6 mature estrous rabbits by intravenous injection of about 50 rat units of human chorionic gonadotropin.

ii. On the fourteenth day, the rabbits are examined for the presence of well-developed mammary glands characteristic of pseudopregnancy.

iii. The lactogenic hormone is injected subcutaneously once daily for 6 days, and the degree of enlargement of the glands with secretion is rated on the seventh day.

iv. For a unit response, an average of *plus three* $(+++)$ rating in a group of 6 animals is required. The repeatability of the method is indicated in Table I.

TABLE I

Relation between the Gardner-Turner Rabbit Unit and the McShan-Turner Pigeon Unit. The 1938 Initial Extract of Cattle Pituitary Was Used[a]

Rabbit No.	Body wt. g.	Amt. injected 100 g. body wt. mg.	Total amt. inj. mg.	McShan-Turner lactogenic U.	Gardner-Turner rating
1	2550	3.5	91.0	227.5	$+++$
2	3430	3.5	119.0	297.5	$++++$
3	2770	3.5	98.0	245.0	$++++$
4	3080	3.5	108.5	271.3	$+++$
5	3590	3.5	126.0	315.0	$++++$
6	3150	3.5	112.0	280.0	$+++$
Av.	3095	3.5	109.1	272.8	3.5
1	4275	2.33	100.3	250.8	$++$
2	2560	2.33	60.7	151.7	$+++$
3	2775	2.33	65.3	163.3	$+++$
4	3000	2.33	70.0	175.0	$++$
5	2720	2.33	63.0	157.5	$+++$
6	3110	2.33	72.3	180.8	$++$
Av.	3073	2.33	71.9	179.8	2.5

[a] From Bergman *et al.* (1940).

C. LOCAL INTRADUCTAL METHOD OF LYONS (1942)

Lyons (1942) suggested a sensitive rabbit assay method which requires no more lactogenic hormone than some of the pigeon crop methods. This method is based on the ability of lactogenic hormone to act directly on mammary tissue. Small amounts of lactogen are injected directly into one or more of the 6 milk ducts of the nipple of a rabbit. A localized lactation appears in the gland sector stimulated. Since a rabbit usually has at least 8 nipples, there are about 48 different sectors available in a single animal for local assays. He found that the minimal effective dose by this localized technic was slightly less than 1 I.U. compared with the minimal effective systemic dose of 75 I.U.

In preparing the rabbits for assay, Lyons used virgin rabbits, 4 to 5 months old, hysterectomized and ovariectomized. To induce growth of the mammary glands, they were injected subcutaneously 5 days weekly for 4 weeks with 20 μg. of estrone and 1 mg. of progesterone. The rest of the procedure is as follows:

i. On the third day after the last injection of the two hormones, the rabbits are anesthesized by the intravenous injection of 200 mg. of nembutal. The hair is shaved over the mammary region.

ii. The nipples are squeezed gently between the thumbs in order to press out any accumulated fluid, and any adhering keratin scale is removed. Sectors from which a few drops of fluid can be expressed are best able to receive and retain the injected hormone. Ducts which are found to be plugged or not completely canalized are abandoned, since these result in an accumulation of fluid between the nipple and the block.

iii. With the aid of a binocular dissecting microscope, single test or control solutions are injected into individual sectors with a 1.0-ml. tuberculin syringe and a 27-gauge hypodermic needle cut down to about 1.0 cm. in length and without a bevel. If the needle pierces the duct epithelium, the injection fluid accumulates around the base of the nipple. In such a case another duct is tried, preferably in another nipple.

iv. A reaction is considered positive if by the fifth day milk can be expressed from the nipple.

Lyons assayed 5 different levels of a lactogenic preparation containing 30 I.U./mg. He injected a single mammary gland in each rabbit with 1.0 cc. of a 2% butanol solution containing a given amount of lactogen. Each rabbit provided its own control glands, at least one of which was injected with 2% butanol. The results are given in Table II.

Meites and Turner (1947, 1948a) have used the Lyons technic to induce localized lactation in mature pseudopregnant and pregnant

rabbits. Pseudopregnancy was induced by the intravenous administration of 50 to 75 I.U. of chorionic gonadotropin, and an intraductal injection of lactogenic hormone was made 15 days later. The rabbits

TABLE II[a]

Mammary Sector Reactions in Oophorectomized Virgin Rabbits Pretreated with Estrone and Progesterone and then Injected Intraductally with Varying Amounts of Lactogenic Hormone in 1.0 cc. of a 2% Butanol Solution[b]

No. of animals	Dosage of hormone		Reactions	
	mg.	Approx. I.U.	−	+
6	0.012	0.37	6	0
6	0.025	0.75	3	3
6	0.05	1.5	5	1
6	0.1	3.0	0	6
6	0.2	6.0	0	6

[a] From Lyons (1942).

[b] At least one other gland of each rabbit was injected with a control solution of butanol and in no instance was lactation induced.

were killed on the twentieth day, and the mammary glands were examined for the presence of milk (see Fig. 1).
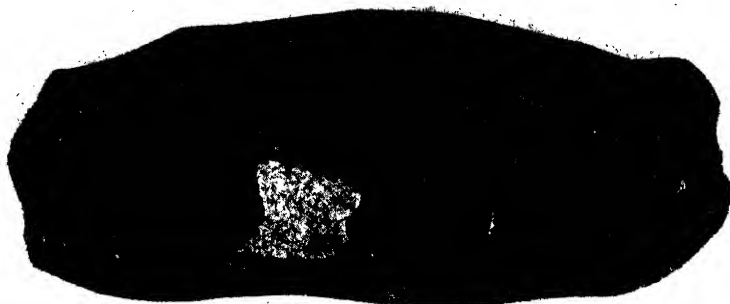
FIG. 1. The localized lactation present in the lower center of this whole mount of rabbit mammary tissue was induced by a single injection of 10 international units (0.33 mg.) of lactogenic hormone* into one duct of the nipple. This mature rabbit was injected on the fifteenth day of pseudopregnancy and killed on the twentieth day.

The advantages of the use of mature pseudopregnant rabbits are (1) the mammary glands are well developed for a lactational response, without the necessity of injecting estrogens and progesterone for a long

* The highly purified lactogenic preparation, which contained 30 I.U. of lactogen per milligram, was kindly furnished by the Schering Corporation, Bloomfield, New Jersey.

period, and (2) the duct openings in the nipple are sufficiently large to see in good light with the naked eye or with the aid of a binocular loupe.

3. Guinea Pig Methods

A. METHOD OF NELSON (1934)

Nelson proposed the following method of assay in guinea pigs:

i. Pregnant guinea pigs are hysterectomized between the fortieth and fifty-fifth day of pregnancy. Care is taken not to disturb the ovarian arteries since loss of blood results in functional castration and the onset of lactation.

ii. After 4 or 5 days of observation the animals are injected twice daily for 7 days.

iii. If milk can be expressed from the nipples during the test period, the result is considered positive. In most cases milk appears in less than 48 hours. Nelson claimed that $2\frac{1}{2}$ times as much lactogen was required to induce lactation in intact as compared to hysterectomized pregnant guinea pigs.

B. METHOD OF LYONS (1941)

i. Lyons proposed the use of multiparous, estrous guinea pigs, one half to $1\frac{1}{2}$ years old, weighing over 650 g. These animals are injected subcutaneously on days 1 to 4 of the estrous cycle, and a unit response is considered to be the smallest amount of lactogenic hormone which will induce lactation in the majority of at least 20 guinea pigs.

ii. For presumptive testing of substances for lactogenic activity, 5 animals per dose suffice. For more exact determinations of unitage not less than 20 animals are used. Lyons stated that animals under 650 g. and those in which injections were begun on days other than 1 to 4 of the cycle did not respond well enough to lactogenic hormone to warrant their use. Acceptable animals were used repeatedly through several estrous cycles provided fatal anaphylaxis did not intervene.

iii. Lyons carried out experiments on 164 multiparous guinea pigs between 650 and 1000 g. to determine the percentage response to 30, 60, 120, and 240 I.U. of lactogenic hormone. In each case the animal was injected for the first time. The 120-I.U. level (at which 56.6% of the animals lactated) was then used to determine the reactivity of these guinea pigs to subsequent injections of the hormone. Excluding approximately 10% of the animals injected for the second series, which died from anaphylaxis, it was found that only 26 of 61 (42.6%) responded to 120 I.U. on the second test, and 25 of 64 (39.1%) responded to the same dose upon being tested for the third time. Thus, fewer animals responded

the second and third time than during the 1st time the animals were injected.

iv. The diminished responsiveness suggested that some animals would probably not be suited for repeated tests. Subsequently, it was found that animals which had proved reactive to not more than 240 I.U. during the first test would respond just as well during the second trial as the first trial. Lyons stated that errors due to variations in responsiveness of guinea pigs in different colonies can be minimized by using a standard preparation. "Proved" animals (those which respond the first time to not more than 240 I.U.) are satisfactory for use from 6 months of age until they reach $1\frac{1}{2}$ years.

III. PIGEON ASSAY METHODS

1. Introduction

Crop sacs of pigeons and doves are thin transparent membranes except during the latter half of the incubation period. At this time in both males and females, they become thickened and for about a 3-week period secrete "crop milk," which they feed to their young. This occurs through stimulation of the animal's own pituitary, and can be induced experimentally in non-incubating pigeons or doves by administration of lactogenic hormone.

The use of the pigeon or dove offers many advantages over mammalian methods: (1) Pigeons require no preliminary preparation, whereas mammals require sufficient development of the mammary gland system to enable them to respond to lactogenic hormone. (2) The results are usually more quantitative and reproducible than mammalian methods. (3) Pigeons are much more sensitive to lactogen than mammals. An extraordinary degree of sensitivity can be obtained by intradermal injections directly over the crop sacs, making it possible to determine the lactogenic hormone content in the pituitaries of small animals and in blood and urine. (4) Contamination with other pituitary hormones does not appear to affect the response to lactogen, particularly when the preparations are injected intradermally over the crop sacs. (5) The response to lactogenic hormone in the two sexes is similar. (6) Pigeons are usually cheaper to purchase than rabbits or guinea pigs.

2. Principal Variants in Pigeon Assay Methods

Probably the principal variants which affect the response of the pigeon to lactogenic hormone are: (a) race, (b) age and weight, (c) season and temperature, (d) route of injection, and (e) injection volume. Each of these factors will be discussed separately.

A. RACE

Riddle and Bates (1939) and Riddle (1947) stated that racial variation in sensitivity to lactogenic hormone may reach a maximum of ten- to twenty-fold and therefore recommended the use of pure strains. Hall (1944) claimed that even within the same race of pigeons (White Carneaux) there may be some change in response to lactogenic hormone from year to year.

B. AGE AND WEIGHT

The area of the unstimulated crop sac was found to be proportional to body weight by Riddle and Bates (1939). These authors also observed that within a race the effect of individual body weight variation was of little significance. McShan and Turner (1936) who used common pigeons in their assay method, noted that light young birds and very heavy birds were less uniform in their response than birds weighing from 260 to 340 g. Reece and Turner (1937) found that when they injected common pigeons intradermally over the crop sacs, mature pigeons were more sensitive to lactogenic hormone than squabs.

C. SEASON AND TEMPERATURE

Bates and Riddle (1941) reported that in 24 groups of 10 young White Carneau pigeons injected at intervals over a period of 2 years, the crop sacs showed a semi-annual cyclic variation in their weight response to a constant dose of the same lactogenic preparations. The greatest response was obtained during winter and summer, and the lowest response during October-November and again during April-May. They concluded therefore, that season is an important factor and should be controlled by a concurrent test with a standard preparation of lactogenic hormone. Folley *et al.* (1940) studied the effects of light and temperature on the pigeon crop gland response; they concluded that light exerts no influence, while an optimal response is achieved at a temperature in the region of 15°C.

D. ROUTE OF INJECTION

The route of injection influences the response markedly. Riddle and Bates (1939) claimed that sub- and intracutaneous injections are 10 times as effective as intravenous injections; 7 to 8 times as effective as intraperitoneal injections; and 4 times as effective as intramuscular injections. Meites *et al.* (1941) found the relationship between subcutaneous, intramuscular and intradermal injections over the crop glands to be of the order of 1:1.25:1/160. Lyons (1937) found that by intra-

dermal injections over the crop gland he could detect one ten-thousandth of the amount of lactogenic hormone required to give a response by the subcutaneous route of injection.

E. INJECTION VOLUME

The volume of injection fluid is primarily of importance when injections are made intradermally over the crop sacs. Bates and Riddle (1940) found that a dilution of 10 times increased the effectiveness of injected lactogen by a factor of 4. Meites *et al.* (1941) reported that two-, three-, and fivefold differences in injection volume did not affect the response to lactogen. Hall (1944) found that doubling the injection volume did not change the response, but when the volumes were increased five- and tenfold there was some increase in response. Although these different results were obtained in different breeds of pigeons and under different assay conditions, it would seem advisable to use a constant volume for intradermal injections over the crop glands.

F. USE OF A STANDARD PREPARATION

Many of the variants inherent in the various pigeon assay methods (also mammalian methods) can be overcome by using a standard preparation of known potency. By injecting the standard preparation into a group of control birds, the potency of the unknown preparation can be directly compared. This is particularly applicable when injecting intradermally over the two crop sacs, since the unknown can be directly compared to the standard in the same bird.

3. Systemic Methods

A. CROP WEIGHT METHOD OF RIDDLE *et al.* (1933, 1939)

In this method Riddle and Bates (1939) recommend that a large number of birds be injected with a selected dose, the potency of which can then be read from a table relating the average crop gland weight to units injected. The authors state that a linear relation exists between the crop weight and the logarithm of dosage over a considerable range. The assay is conducted as follows:

i. Selected birds 2 to 3 months old are injected intramuscularly (intrapectorally) once daily for 4 days.

ii. On the fifth day, or about 96 hr. after the first injection, the birds are killed.

iii. The stimulated area of each crop sac is cut, cleaned, and weighed. The procedure recommended is "to make a midventral incision through the skin and crop wall from keel to head and remove the contents of

the crop and adhering crop-milk. The two lateral pouches are then cut around, removed, the fat cleaned from the back of the glands and the glands weighed. Thus the unstimulated tissue in the dorsal midline and that around the proximal and distal opening of the crop are never weighed."

iv. The unit is the extrapolated value for the threshold dose in an average sample of birds. Crop sacs weighing 1150 mg. represent 10 units of lactogenic hormone.

B. MINIMUM RESPONSE METHOD OF LYONS *et al.* (1933, 1937)

Lyons and Catchpole (1933) found that it was unnecessary to depend on weight increases in the crop glands over that of controls, since they could observe beginning growth changes in crop glands weighing less than the controls. The assay is conducted as follows: .

i. One-month-old Silver King squabs are injected subcutaneously daily for 4 days.

ii. The birds are killed on the fifth day, and the entire crop sacs are removed and examined by transmitted light.

iii. A unit is considered the smallest amount of hormone which gives a positive response in a majority of 5 birds. Positive stimulation is indicated by the presence of typical parallel strands of thickened mucosa.

iv. Lyons showed that when dosage was plotted against crop weight, a fairly straight line resulted, even though only 3 animals were used at each dosage level. Such a curve is helpful in determining the approximate potency of preparations.

C. MINIMUM RESPONSE METHOD OF MCSHAN AND TURNER (1936)

These workers devised an assay method based upon the production of minimal crop proliferation in common pigeons. This method, based on a 50% level of response in 20 pigeons, was found to be more accurate than the weight method. The assay is conducted as follows:

i. Twenty common pigeons of both sexes, weighing between 260–340 g. are used. Birds which are too light or too heavy are less uniform in their response.

ii. The lactogenic preparation in injected daily for 4 days just beneath the skin into the breast muscle (see Fig. 2).

iii. On the fifth day or about 96 hr. after the first injection the birds are killed and the entire crop sacs are removed and examined by transmitted light for the presence of proliferation. Inasmuch as there may be a difference of opinion among unexperienced workers as to whether a

minimal response is present, it is suggested that two observers examine the glands independently.

iv. The assay unit is defined as "the total amount of hormone injected during a period of 4 days which will cause a minimum but definite proliferation of the crop glands of $50 \pm 11\%$ of 20 common

FIG. 2. Technic of shallow intrapectoral injection of lactogenic preparation into a pigeon.

pigeons weighing 300 ± 40 g." The relation between hormone dosage and percentage of birds showing positive responses is given in Fig. 3 (McShan and Turner, 1936).

Li and Evans (1948) employed this method routinely to estimate lactogenic potency, and found that satisfactory results could be obtained using only 3 birds per group. If 2 out of the 3 birds gave a positive response, the amount of hormone used was considered to be one unit.
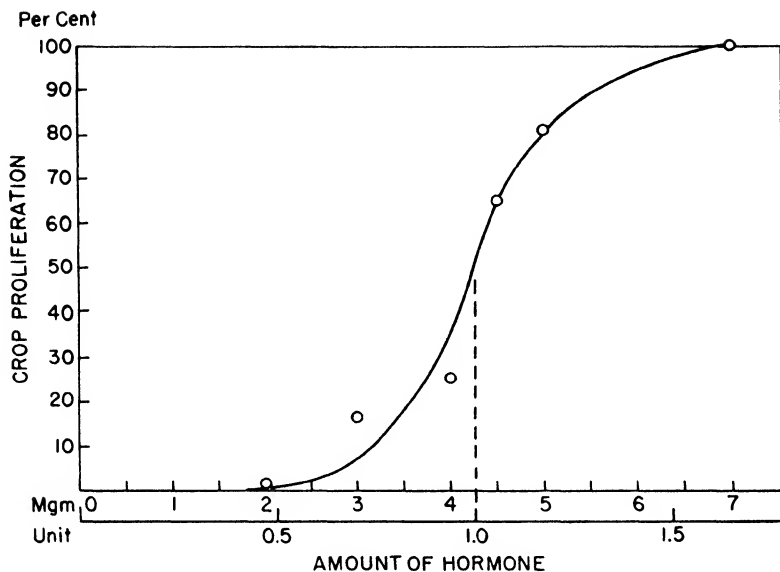
Fig. 3.   Relation between amount of hormone injected and percentage of pigeons showing crop gland proliferation.   Relation is described by a characteristic sigmoid curve which indicates extreme sensitivity to small amount of hormone in region of 50% response.   The pigeon unit is based upon this curve, one unit of the hormone being the amount required to give a 50% response.   (From McShan and Turner, 1936.)

4. Local ("Micro") Methods

A. METHOD OF LYONS AND PAGE (1935)

Lyons and Page discovered that when lactogenic hormone was injected intracutaneously over the crop sac, only a small area directly over the site of injection was stimulated.   This is the most sensitive of all the known methods of assaying lactogen.   It is necessary to inject so as to obtain a bleb or blister, since if the injections are subcutaneous instead of intracutaneous the sensitivity is largely lost.   By this method it is possible to compare directly two different preparations, i.e., a standard and unknown, since injections can be made over each crop sac of the same bird.

Except for the site of injection, the procedure is the same as described for the minimum stimulation method of Lyons et al. (1933, 1937).   Before beginning the assay, the feathers are plucked from the crop sac area of the squabs.   Four daily intradermal injections are made with a 27-gauge hypodermic needle.   The injections are facilitated when the crops are distended with food.   The follicles from which the feathers have been plucked are the best injection sites (see Fig. 4).

Lyons (1937) claimed this "micro" technic enabled him to detect one ten-thousandth of a micro unit. Both Lyons (1937) and the authors (1940) have used this method for the assay of minute amounts of lacto-

FIG. 4. Technic of intradermal injection of lactogenic preparation over a crop sac. Note the "bleb" formed at the site of injection. A direct comparison of the potencies of two different preparations can be made by a similar injection over the other crop sac.

genic hormone in human urine. The authors (1942) also employed this technic to assay the lactogenic hormone in the blood of rabbits.

B. METHOD OF BERGMAN, MEITES, AND TURNER (1940)

The procedure is the same as described for the McShan-Turner (1936) method, except that the injections are made intradermally over the crop sacs instead of intramuscularly. The unit is defined as that amount of hormone which when injected intradermally over the same area of the

crop glands of 20 common pigeons (weight 300 ± 40 g.) will elicit a minimum response in 50 ± 11% of the pigeons. This method is highly quantitative (see Table III).

TABLE III

Percentage of Minimal Crop Gland Responses to Different Dosages of Lactogenic Hormone Injected Intradermally over Crop Sacs[a]

No. pigeons in group	Lactogenic preparation	Amount injected per pigeon mg.	No. of positive responses	Percentage response	
10	Int. Standard	0.000416	0	0	
10	" "	0.000520	0	0	
10	" "	0.000624	5	50	
20	" "	0.000624	9	45	unit range
20	" "	0.000624	11	55	
20	" "	0.000624	9	45	
10	" "	0.000624	5	50	
20	Cattle pit. extract	0.001	20	100	
20	" " "	0.002	11	55	
20	" " "	0.002	10	50	unit range
20	" " "	0.002	8	40	
20	" " "	0.002	9	45	

[a] From Bergman *et al.* 1940, and Meites *et al.* 1941.

C. METHOD OF REECE AND TURNER (1937)

This method has proved very useful for the assay of pituitaries of small laboratory animals, although it probably will not prove as quantitative in most hands as the pigeon assay methods previously described. Ratings from 0.25 to 4 are given each of the stimulated crop glands, leaving room for some degree of subjectivity. Since its relation to several other pigeon assay units is known (see Table V), it can be used as a preliminary test for more quantitative procedures. It is recommended that a standard preparation of known potency be injected over one crop sac in each pigeon and the unknown preparation be injected over the other crop sac for comparative purposes. The assay is carried out as follows:

i. If the pituitary glands of small laboratory animals are to be assayed, they are first macerated into a paste with a small agate mortar and pestle and suspended in a small but definite amount of distilled water. The suspension is taken up with a small syringe and a number 27-gauge needle.

ii. The pituitary suspension is injected daily for a period of 4 days, intradermally over the same area of the crop gland in 0.1 ml. volumes with a 27-gauge needle.

iii. On the fifth day the birds are killed, the complete crop glands are removed, and each gland is rated for degree of proliferation. The total of the ratings in all the crop glands represents the units of lactogen in the preparation administered.

iv. The bird unit is defined "as that amount of hormone which will cause the proliferation of an area of the crop gland about the size of a nickel when injected intradermally over the crop gland of a mature pigeon for 4 days, the bird being sacrificed upon the fifth day." The

TABLE IV

Range of Accuracy and Repeatability of Reece-Turner Assay Method[a]

No. pigeons in group	Lactogenic preparation	Amount injected per pigeon mg.	Average rating per gland
5	No. 4-fresh pit.	4.0	2.6[b]
10	No. 4-fresh pit.	1.5	2.0
10	No. 4-fresh pit.	0.8	1.0
5	No. 5-fresh pit.	4.0	3.1[b]
10	No. 5-fresh pit.	1.0	1.9
10	No. 5-fresh pit.	0.5	1.0
5	No. 6-fresh pit.	4.0	3.0[b]
10	No. 6-fresh pit.	1.0	2.1
10	No. 6-fresh pit.	0.5	1.3
10	1938 cattle pit. extract	0.025	1.4
20	1938 cattle pit. extract	0.020	1.2
20	1938 cattle pit. extract	0.018	1.0
20	1938 cattle pit. extract	0.018	1.0
20	1938 cattle pit. extract	0.018	0.9
10	1939 cattle pit. extract	0.025	1.5
20	1939 cattle pit. extract	0.020	1.2
20	1939 cattle pit. extract	0.016	1.0
20	1939 cattle pit. extract	0.016	1.0
20	1939 cattle pit. extract	0.016	0.9

[a] From Meites and Turner (1948a).

[b] It can be seen that when amounts of hormone which induce crop ratings greater than 2 are given, the response is no longer proportional to dosage.

proliferation of an area of 2 cm. in diameter is considered to be equivalent to 2 units.

Reece and Turner (1937) found that the response was directly related to dosage, up to a rating of 4 (an area of proliferation of 4 cm. in diameter). It has been the experience of the senior author, however, that crop gland responses greater than 2 are difficult to rate, and that the most quantitative results can be achieved by injecting only enough hormone to induce a rating of 2 or less (see Table IV).

IV. COMPARISON OF ASSAY METHODS USING INTERNATIONAL STANDARD LACTOGENIC HORMONE

Bergman *et al.* (1940) and Meites *et al.* (1941) compared several mammalian and pigeon assay methods, first using lactogenic extracts prepared in the Missouri laboratory and later the international standard lactogenic preparation.

The 3 pigeon assay methods used were the same except for route of injection; namely, (1) shallow intrapectoral (McShan-Turner method), (2) subcutaneous over the pectoral muscles and (3) intradermal over each crop gland (Bergman *et al.*). The unit for the assays was defined as the total amount of hormone injected during a period of 4 days which will cause a minimum but definite stimulation of the crop glands of 50 ± 11% of 20 common pigeons weighing 300 ± 40 g. The crop glands in all cases were examined by two observers using transmitted light. The comparison of the crop gland assays is given in Table V.

TABLE V

Comparison of Crop Gland Assays Using I.S. Lactogen (1 I.U. = 0.1 mg.)[*]

No. pigeons in group	Amt. of I.S. lactogen injected per pigeon mg.	Injection route	Total volume injected cc.	Positive response	Percentage response
10	0.15	Subcutaneous	0.40	8	80
30	0.125		0.40	23	76
20	0.11		0.40	14	70
20	0.10		0.40	9	45 ⎫
20	0.10		0.40	11	55 ⎬ unit range
20	0.10		0.40	11	55 ⎭
20	0.125	Shallow	0.40	10	50 ⎫ unit
20	0.125	intrapectoral	0.40	11	55 ⎭ range
10	0.000416	Intradermal	0.40	0	0
10	0.000520	(micro)	0.40	0	0
10	0.000624		0.40	5	50 ⎫
20	0.000624		0.20	9	45 ⎪
20	0.000624		0.40	11	55 ⎬ unit range
20	0.000624		0.60	9	45 ⎪
10	0.000624		1.00	5	50 ⎭
10	0.000832		0.40	10	100

[*] From Meites *et al.* (1941).

For the mammalian lactation test, the Bergman *et al.* (1940) modification of the Gardner-Turner (1933) rabbit assay method was used. A total of 34 rabbits were assayed with the same preparations used for

the pigeon assays. Lyons (1941) had already determined that his guinea pig unit required 80 international units of lactogenic hormone.

The comparison of lactogenic hormone requirements in terms of international units for the various mammalian and pigeon assay methods is given in Table VI. The international standard lactogen was reported

TABLE VI

Comparison of Lactogenic Assay Units in Different Animals[a]

Assay unit	Assay animal	No. of assay units per I.U.	No. of I.U. per assay unit
Subcutaneous unit	Common pigeon	1.00	1.00
Shallow intrapectoral unit		0.80	1.25
Intradermal unit (micro test)		160.00	0.00625
Reece-Turner intradermal unit	Common pigeon	22.2	0.045
Riddle-Bates intrapectoral unit	Specific strains of doves and pigeons	1.00	1.00
Lyons subcutaneous unit	Silver King squabs	0.60	1.66
Gardner-Turner rabbit unit	Mature female New Zealand white rabbits	0.00294	340.00
Lyons guinea pig unit	Nulliparous female guinea pigs weighing 650–1000 g.	0.0125	80.00

[a] From Meites *et al.* 1941.

to contain 10 Riddle-Bates or international units per milligram, which was the same as found in our "subcutaneous unit." On the basis of a private communication from Lyons (1940), it was deduced that his "subcutaneous unit," using Silver King squabs, was equal to 0.60 of an international unit.

V. Comparison of Lactogenic Hormone Content in Pituitaries of Various Mature Animals

A comparison of lactogenic potency in the pituitaries of various sexually mature animals is given in Table VII. It can be seen that, with the exception of the male guinea pig, the pituitaries of the female animals contain much more lactogen than the males. It is interesting that this same sex difference appears in pigeons, despite the fact that the male pigeon also secretes crop milk during normal incubation and brooding and its crop gland is as sensitive to the administration of lactogen as the female pigeon.

On the basis of hormone concentration within the pituitary, the female goat ranks first, the dairy cow second, and the male and female guinea

pig are third and fourth respectively.   On a 100-g. body weight basis, the female rat pituitary contains the most lactogen, the female mouse is second, and the male and female guinea pig are third and fourth respectively.

Chance *et al.* (1939) compared the lactogen content per unit pituitary weight in several species and found the following descending order of

TABLE VII
Comparison of Lactogenic Hormone in Pituitaries of Mature Animals

| Species | No. | Sex | Body weight g. | Pituitary weight mg. | Reece-Turner Lactogenic Units | | | | |
					Per pit.	Per mg. pit. tissue	Rank	Per 100 g. body weight	Rank
Mouse[a]	30	F	21.3	1.80	0.42	0.22	7	1.98	2
Mouse[a]	48	M	28.3	1.85	0.27	0.13	9	0.92	6
Rat	10	F	220.0	10.10	4.76	0.47	5	2.16	1
Rat	21	M	221.0	5.50	0.89	0.16	8	0.40	8
Guinea pig	11	F	585.0	15.16	9.79	0.64	4	1.67	4
Guinea pig	8	M	598.0	16.14	11.53	0.71	3	1.93	3
Cat[b] (anestrus)	12	F	1858.0	20.00	1.31	0.06	10	0.07	11
Cat[b]	7	M	2233.0	23.80	0.89	0.03	11	0.03	13
Rabbit	6	F	3087.0	38.80	10.00	0.25	6	0.32	9
Rabbit	16	M	3065.0	28.08	1.55	0.05	10	0.05	12
Goat		F				1.33	1	1.66	4
Dairy cow[b]		F		1801.8	1449.3	0.80	2	1.49	5
Beef cow[b]		F		1548.6	746.9	0.48	5	0.90	6
Common pigeon	8	F	276.0	4.32	2.0	0.46	5	0.72	7
Common pigeon	16	M	302.0	3.92	0.6	0.15	8	0.20	10

[a] Data from Reece and Turner, 1937.
[b] Data from Hurst and Turner, 1942.

potency: sheep and cattle, human, and horse and pig.   This would seem to be in agreement with the earlier report of Bates *et al.* (1935) that beef and sheep pituitaries contain about 20 times as much lactogen as swine pituitaries.   Reece and Turner (1937) found that the pituitaries of dairy cattle contained considerably more lactogen than beef cattle, but Hall (1944) was unable to corroborate this observation.

VI. COMPARISON OF LACTOGENIC HORMONE CONTENT IN PITUITARIES OF VARIOUS ANIMALS AS AFFECTED BY PREGNANCY AND LACTATION, SUCKLING, AND FOLLOWING ESTROGEN STIMULATION

The lactogen content of the pituitaries of various animals during pregnancy and lactation are presented in graphic form in Fig. 5.   A
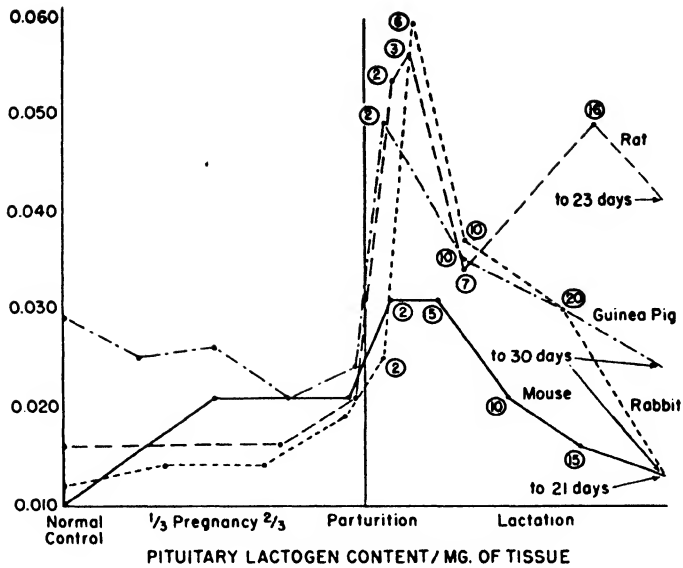
FIG. 5. Lactogenic hormone content per milligram of pituitary tissue for the various species. Encircled figures indicate days postpartum. A rapid postpartum rise in lactogen content per milligram of pituitary tissue is clearly indicated. (From Hurst and Turner, 1942.)
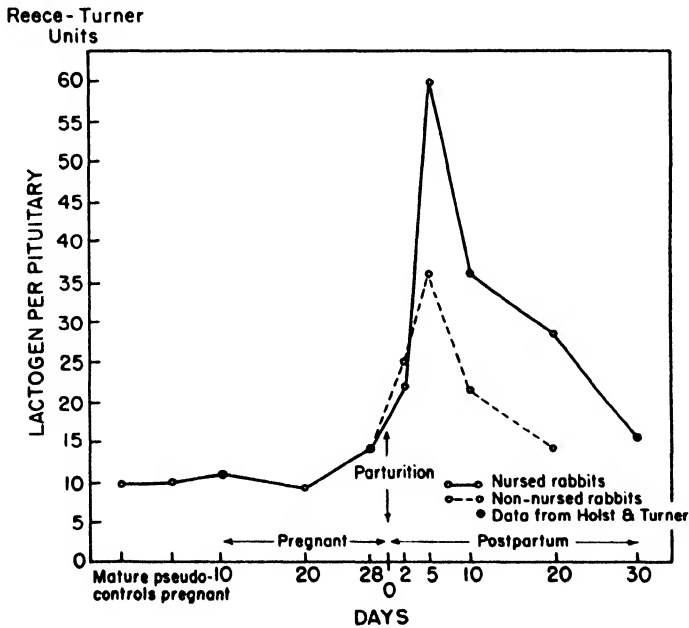
FIG. 6. Graph showing changes in lactogenic hormone content of the pituitary during pregnancy and lactation. Note the higher pituitary lactogen content of the nursed rabbits as compared to the non-nursed rabbits. (From Meites and Turner, 1942a.)

sharp rise occurs in all these species immediately after parturition. With the exception of the mouse there is no marked increase in pituitary lactogen content during pregnancy. The slight rise which has been found in rats, rabbits, guinea pigs, and goats just prior to parturition is believed to be significant, however, since some secretory activity in the mammary glands is usually initiated at this time. The full inflow of milk does not normally occur until after parturition.
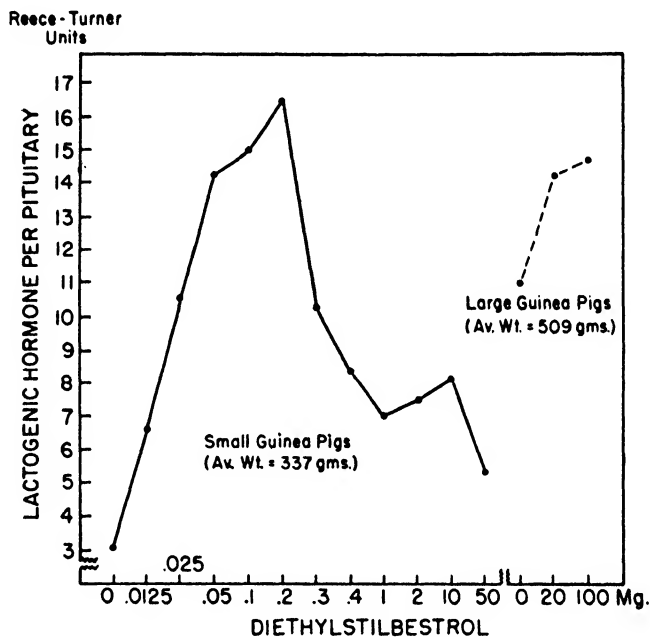
FIG. 7. Relation of dosage of diethylstilbestrol injected over a 5-day period to increase in pituitary lactogen content of male guinea pigs. The increases obtained with 0.05 to 0.20 mg. of diethylstilbestrol is equal to that present in parturient, lactating guinea pigs. (From Meites and Turner, 1942.)

The role of the suckling influence during lactation on the lactogen content of the rabbit pituitary is shown in Fig. 6. These pituitaries were assayed by the Reece-Turner method (1937). It can be seen that with the exception of the second day postpartum, the pituitaries of the suckled rabbits contained considerably more lactogenic hormone per pituitary than the non-suckled rabbits.

The effects of injecting increasing levels of diethylstilbestrol on the pituitary lactogen content of guinea pigs is shown in Fig. 7. These results, which have been duplicated in other species as well (Reece and Turner, 1937; Meites and Turner, 1948a, b), are believed to be significant

in view of the authors' theory that estrogen is responsible for the normal initiation of lactation at parturition (1942b, 1948a).

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Mammogenic Hormone

By C. W. TURNER

CONTENTS

I. INTRODUCTION

In spite of the intensive research devoted to the study of the hormones influencing the growth of the mammary gland during the past 20 years, there still rages considerable controversy over the extent of the involvement of the anterior hypophysis. In the 1920's and early 1930's, the role of estrogen in stimulating the growth of the duct system and of progestin in combination with estrogen in stimulating the growth of the lobule-alveolar system of males and castrate females appeared to offer a reasonably satisfactory explanation (Turner, 1932–39).

However, in 1936 my students began to secure evidence of the necessity of the intact pituitary in order to secure mammary growth with the above gonadal hormones (Reece *et al.*, 1936; Gomez and Turner, 1936; and Gomez *et al.*, 1937). These and other observations led us (Gomez and Turner, 1937, 1938) to advance the theory that estrogen stimulated the pituitary to secrete an increased amount of a factor which stimulated duct growth while estrogen plus progestin stimulated the pituitary to secrete an increased amount of a factor which causes lobule-alveolar

growth. These pituitary factors were named *mammogen* I, the duct growth factor, and *mammogen* II, the lobule-alveolar growth factor.

In the following years, while many observers confirmed our observations concerning the lack of material duct or lobule-alveolar growth in the absence of the anterior hypophysis, others claimed from slight to normal gland growth (see Trentin and Turner, 1948, for a review of literature). Slight autonomous mammary gland growth may be stimulated in hypophysectomized animals just as the other hypophyseal hormone target glands such as the thyroid (Leblond and Sue, 1941; Leblond and Gross, 1948), adrenal cortex (Deane and Greep, 1946), and gonads (Chu, 1940) may function to a limited extent.

Evidence of a positive nature of the role of the anterior lobe in mammary gland growth was furnished by the demonstration that the administration of hypophyseal tissue would cause mammary gland growth in hypophysectomized or gonadectomized animals. A review of the earlier work and presentation of data indicating the gland duct stimulating action of anterior lobe tissue and extracts was presented by Lewis and Turner (1939). Later Mixner and Turner (1943) developed an assay method for the mammary lobule-alveolar growth-stimulating properties of these tissues. Recently, Trentin and Turner (1948) presented evidence indicating that these two assay methods are measuring the same anterior pituitary factor.

Attempts to extract and purify the mammary growth stimulating factor of the anterior lobe have been only partially successful. The early attempts at extraction by Lewis and Turner (1939) resulted in the apparent separation of an active fraction by lipid solvents. It was concluded, therefore, that the factor was distinct from the protein pituitary hormones. Since assays of fresh pituitary and active extracts for estrogen and progesterone yielded only isolated positive results, it was thought that the positive stimulation of the mammary gland was not due to the presence of these hormones in the anterior hypophysis.

As a result of further research by Trentin and Turner (1948) using repeated extraction of the various fractions, it was concluded that the active factor was present in the protein fraction rather than in the fraction extracted with lipid solvents. In the crude separation of lipid and protein in the earlier work, it was quite possible that some proteinaceous material was carried over into the "lipid" fraction. An account of the present status of extraction procedure will follow a description of the assay methods and a study of the amount of mammogenic hormone present in the anterior hypophyses of animals in various stages of reproduction.

For a review of the normal growth of the mammary glands during

recurring estrus cycles and pregnancy the reader is referred to Turner (1939). Briefly, the virgin animal develops a more or less extended duct system which upon conception is rapidly converted by side branches and alveolar growth into a fully grown mammary gland. These stages of development may be observed most clearly by the preparation of whole mounts of the glands. Since the examination of the glands by this method is an integral part of the assay methods to be described, it will be presented in detail.

II. Preparation of Whole Mounts of Mammary Glands

In the study of early stages of mammary duct or lobule-alveolar growth, whole mounts of the glands rather than sections have advantages (Turner and Gomez, 1933). Stages of development may be observed by removal of gland biopsies. This may be accomplished by the removal of a triangular piece of the subcutaneous fatty tissue fanning out laterally from the teat. This tissue containing the mammary gland is dissected out, pinned on a flat cork and fixed as indicated below.

The mammary glands of mice are prepared for examination as follows. The skin is slit down the center of the back and removed together with the subcutaneous fascia, pinned out on flat sheets of cork and fixed in Bouin's solution for 12 to 24 hr. The subcutaneous fascia containing the glands is scraped from the skin, washed in water, stained in Mayer's hematoxylin and the mammary glands examined with a binocular dissecting microscope while differentiating in 70% acid-alcohol. If the glands are to be preserved they may be dehydrated in alcohol, cleared in xylol, and mounted in balsam on slides of suitable size (Gomez and Turner, 1937; Mixner and Turner, 1943).

Sections of glands for histological study are removed from the skin (but not trimmed), washed, dehydrated, cleared, and embedded in paraffin. Sections may be cut from 6 to 10 μ in thickness, stained in Delafield's hematoxylin and eosin or Mallory's connective tissue stain.

III. Assay of Duct Growth

Early studies of the hormones stimulating mammary duct growth were qualitative. In order to determine the amount of mammary gland stimulating factor in the anterior hypophysis, it became essential to develop a more quantitative assay method. The observation of Turner and Gomez (1933), confirmed by Gardner et al., (1934) that the mammary glands of male albino mice remain rudimentary throughout life, suggested the desirability of the male mouse as an assay animal. Examination of 164 normal young male mice weighing 15 to 25 g. by Lewis and Turner

(1939) showed no rudiments consisting of more than a few primary ducts with perhaps a few short side stubs.

Lewis and Turner (1939) outlined a duct growth assay method using normal male mice. A mouse unit of the mammogenic duct growth factor was defined as *the amount of tissue or extract required (per mouse) to produce definite signs of development in one or more glands of 50 ± 10% of at least 10 male albino mice weighing 15 to 25 g.* Injections were made once daily, subcutaneously, for 6 successive days, and the animal sacrificed on the seventh day. Minimal signs of mammary rudiment development were considered to be the thickening of the main ducts with definite

Male mice weighing 15–25 g. are used.

Control gland, very rudimentary.

Stimulated gland growth of ducts with enlarged end buds.

Injected subcutaneously once daily for 6 days. Glands removed on 7th day, fixed, stained, and examined for evidence of growth of ducts.
Mouse unit —amount of tissue or extract necessary to secure 1 or more glands showing growth stimulation in 50% ± 10% of ten male mice.

FIG. 1. Assay of mammogenic duct growth factor.

proliferation of secondary branches which usually show enlarged, dark staining end buds.

1. Precautions with the Assay Method

The earlier observations of male mice indicated that their mammary glands remain rudimentary throughout life. However, Richardson and Cloudman (1947) have recently reported that male mice of certain strains show considerable extension of the mammary duct system. Trentin and Turner (1948) reported that certain strains of mice were unsuitable for use in the assay of the duct growth factor due to spontaneous development of the ducts in varying percentages of the animals.

It is important in this assay that control mice be examined for the extent of duct development. Only those strains should be used whose ducts remain rudimentary throughout life.

A second source of variation in assay results may come as a result of strain variation in responsiveness to the anterior hypophyseal hormone. Strains with the greatest sensitivity are desirable as the requirements for response will be less.

2. Other Uses for the Assay Method

In addition to the assay of pituitary preparations, this method provides a quantitative measure of the capacity of estrogenic compounds to stimulate mammary duct growth. The writer would interpret the assay of the estrogens as measuring their capacity to increase the permeability of the vascular bed in the region of the growing ducts. It would also be possible to combine estrogen with pituitary preparations to measure their synergism.

3. Assay of Cattle Anterior Hypophyses

Using the assay method just described, Lewis and Turner (1939) determined the duct growth stimulating potency of a series of cattle pituitaries of various types. It is believed to be of considerable significance that the administration of cattle pituitary preparations stimulated the growth of the duct system. In the light of the recent conclusion that the mammogen duct growth and lobule-alveolar growth factors are identical, the observation of the rise in the mammogen content of the pituitaries with the advance of pregnancy during the period of rapid lobule-alveolar growth and a decline in late pregnancy becomes rationalized.

IV. Assay of Lobule-Alveolar Growth

Mixner and Turner (1943) developed an assay method involving the stimulation of the growth of the mammary lobule-alveolar system comparable to the type of growth occurring normally in pregnant or pseudo-pregnant mice. Nulliparous mice weighing 12 to 18 g. were ovariectomized. A piece of mammary gland was removed, fixed, stained, and examined to insure that a smooth duct system was present. Injections of the material to be assayed were started immediately to insure satisfactory results.

A mouse unit of the mammogenic lobule-alveolar growth factor was defined as *the amount of material required per mouse injected over a period of 10 days to obtain minimal mammary lobule-alveolar growth in 50 ± 10% of 10 or more castrate nulliparous female mice when a total of 75 I.U. of estrone was simultaneously injected.*

The need of estrogen in the assay was explained as follows. Although anterior pituitary tissue or extracts will directly stimulate mammary gland growth, the requirement is about 5 times that when estrogen is administered. The estrogen is believed to act directly on the stromal tissue surrounding the mammary gland producing an increased hyperemia

and vascularity associated with an increased permeability of the capillary bed.

By this action of estrogen, more rapid growth of the mammary gland is promoted indirectly by increasing the amount of pituitary mammogen in the intercellular spaces in the region of the mammary gland and also by increasing the amount of nutrients available to the growing gland (Mixner and Turner, 1942).

The first easily detectable signs of alveolar formation in pregnant mice consisted of the proliferation of numerous side stubs and flowery ends on short interlobular ducts. This stage was taken as the criterion
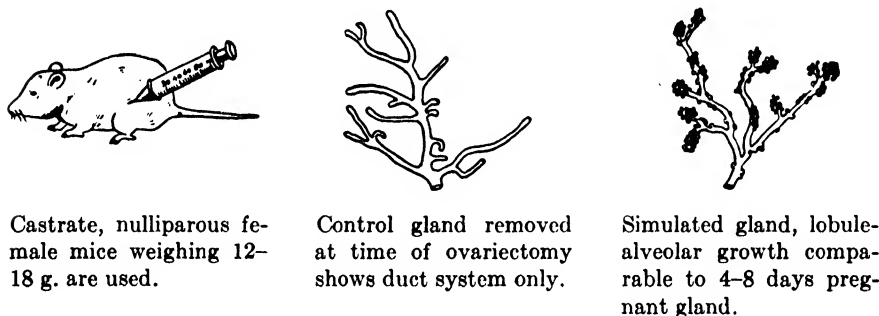
Castrate, nulliparous female mice weighing 12–18 g. are used.	Control gland removed at time of ovariectomy shows duct system only.	Simulated gland, lobule-alveolar growth comparable to 4–8 days pregnant gland.

Mice injected subcutaneously once daily for 10 days with assay material. Simultaneous daily injection of 75 I.U. of estrone. Glands removed on the 11th day, fixed, stained and examined for lobule-alveolar growth.

Mouse unit—amount of material required per mouse to obtain lobule-alveolar growth in 50% ± 10% of ten castrate nulliparous female mice when estrone is simultaneously injected.

FIG. 2. Assay of the mammogenic lobule-alveolar growth factor.

of minimal alveolar development for positive results. It is similar to the condition present at 4 days of pregnancy.

1. Strain Variation in Mammary Response

Mixner and Turner (unpublished) have observed strain differences in female mice in the responsiveness of the mammary glands to either gonadal or anterior hypophyseal hormones. Some strains require twice or more the amount of anterior pituitary extract required by other strains to give a 50% response. Further, in 2 strains equally sensitive to pituitary extracts, a difference was observed in the response to progesterone and estrogen.

Since the requirements of anterior hypophyseal tissue for mammary response is rather large at best, it is important to select the most sensitive strain for assay purposes. This can be accomplished by comparing their responsiveness to progesterone and to standard pituitary preparations.

2. Mammogen Content of Anterior Pituitary Preparations and Extracts

Mixner and Turner (1943) assayed a number of cattle pituitary preparations for the lobule-alveolar growth factor. The total dosage required to give a unit response is presented for a number of preparations (Table I).

TABLE I

Assay of Cattle Pituitary for Alveolar Growth Factor

Preparation	Total dosage mg.	No. of mice	Positive response %
Stockyard run of cattle			
Fresh pituitary	50	13	61.5
Acetone-ether dried	20	13	61.5
Initial extract	7.5	35	51.4
Pregnant cattle			
Fresh pituitary (Lot 1)	35	13	53.8
Acetone-ether dried (Lot 1)	15	13	46.1
Fresh pituitary (Lot 2)	35	13	46.1
Acetone-ether dried (Lot 2)	15	14	57.1
Cattle lactogenic 39	10	12	41.7
Cattle initial 41	10	12	33.3
Schering-lactogenic 46	10	10	50.0
Schering-lactogenic 48	7.5	11	54.5

The assay of anterior pituitary preparations for the mammogen alveolar growth factor indicated a concentration of potency in preparations also rich in the lactogenic hormone. The question arose whether mammogen and lactogen were identical hormones. The observations that the lactogenic hormone remains at a relatively low level during most of pregnancy but rises sharply at parturition (see review by Meites and Turner, 1948) whereas the mammogenic hormone increases with the advance of pregnancy but declines at the approach of parturition (Lewis and Turner, 1939) would appear to rule out this possibility. However, in order to study this problem further, it was decided to compare the mammogenic and lactogenic hormone contents of a series of 14 preparations. The results are shown in Table II. It may be seen that the international units of lactogen per mammogenic lobule-alveolar unit range from approximately 2 to 352, a differential of 176 times. It was concluded that the lactogenic and mammogenic hormones are not identical.

If the lactogenic hormone does possess mammary growth stimulating properties sufficient to replace the losses of the alveolar epithelial cells

during lactation, then it meets the definition of a pituitary mammogen as originally postulated, since its secretion is stimulated by estrogen. It would explain the observations of Turner and Reineke (1936) showing that the continuation of milking on one side of the udder (and the maintenance of secretion of the lactogenic hormone) prevented the rapid involution of the lobule-alveolar system in the unmilked half which occurs when milking is stopped on both sides of the udder.

TABLE II

Comparative Mammogenic and Lactogenic Hormone Assays on a Group of Anterior Pituitary Preparations

Preparation	Mg. extract per mammogenic unit	I.U. of lactogen per mg. of extract	I.U. of lactogen per mammogenic unit
Lot 14, fresh anterior pituitary, pregnant cattle	35.0	0.06	2.1
Lot 15, fresh anterior pituitary, pregnant cattle	35.0	0.06	2.1
Lot 13, fresh anterior pituitary, stockyard run of cattle	44.0[a]	0.06	2.6
Lot 14, acetone-ether dried anterior pituitary, pregnant cattle	15.0	0.3	4.5
Lot 15, acetone-ether dried anterior pituitary, pregnant cattle	15.0	0.3	4.5
Lot 13, acetone-ether dried anterior pituitary, stockyard run of cattle	17.6[a]	0.3	5.1
Lot 13, initial extract of anterior pituitary, stockyard run of cattle	7.5	4.5	33.8
Cattle lactogenic 39	10.0	4.0	40.0
Schering 46-51-p_x-4	10.0	12.0	120.0
CI$_3$ 41-70	13.5	12.0	162.0
Schering 48-hy-ex.4	7.5	30.0	225.0
L-la-40	20.0	12.0	240.0
L-1-41-60	15.0	18.0	270.0
L-1-41-70	17.6[a]	20.0	352.0

[a] Corrected to basis of 50% positive responses from 61.5%.

Similar comparisons were made between the mammogen, thyrotropic and gonadotropic hormones. The chick units of thyrotropin (Bergman-Turner method) per mammogen unit varied from 1.31 to at least 100, a difference of at least 80 times, while the chick units of gonadotropin varied from 0.095 to at least 40, a differential of at least 400 times. It is thus rather apparent that the mammogenic factor is not associated with either the thyrotropic or gonadotropic hormones.

3. Comparison of Duct and Alveolar Stimulating Activities of Various Pituitary Preparations

When Trentin and Turner (1948) discovered that the mammary duct as well as the lobule-alveolar growth factors were present in the protein fraction, the question of their similarity was raised. It was therefore decided to assay a number of anterior pituitary preparations of varying nature and make a comparison of the amount of each extract required to give an assay response by the two methods.

It will be noted that the ratio of the duct unit to the alveolar unit varies from a minimum of 0.5 to a maximum of 1.8 (Table III). It was concluded that the 2 assay methods were measuring the same factor rather than differentiating between a specific duct and a specific alveolar stimulant.

TABLE III

Comparison of Mammary Duct and Alveolar Stimulating Potency of Various Anterior Pituitary Preparations

Preparation	Mg. per duct unit	Mg. per alveolar unit	Alveolar units per duct unit
Fresh anterior pituitary unselected cattle Lot No. 41	50.0	44.0[a]	1.10
Fresh anterior pituitary non-pregnant cattle, Lot No. 16	50.0	30.0	1.70
Acetone dried anterior pituitary, pregnant cattle Lot No. 14	25.0[a]	15.0	1.70
Acetone dried anterior pituitary, unselected cattle, Lot No. 41	15.0	17.6	0.85
Initial extract of anterior pituitary, unselected cattle, Lot No. 41	6.4[a]	7.5	0.85
Cattle lactogenic 39	17.7[a]	10.0	1.80
CI₃ 41-70	7.5	13.5	0.55
Lac. 1a-40	10.0	20.0	0.50

[a] Corrected to 50 % response.

Further evidence for the unity of the mammogenic hormones was presented by Trentin and Turner (1948). It was shown that the sequence of response to estrogen, estrogen plus progesterone, progesterone alone, or anterior pituitary tissue or extracts was the same, namely, first end-bud formation and second alveolar development. The significant difference observed was rather one of rate of development, the estrogen and progesterone treated group reaching the alveolar stage more quickly than

the estrogen treated group.   In the case of pituitary extracts also alveolar
development is never induced without first inducing duct growth.

Further, evidence was reported suggesting that the mammary
alveolar response to estrogens in certain species and at certain dosages
may be dependent upon the ability of estrogen to stimulate the adrenal
cortex and of the adrenal cortex to secrete steroids either identical with
progesterone or resembling progesterone in its ability to synergize with
estrogen in the stimulation of mammary alveolar development.

4. Other Uses for the Assay Method

In addition to the assay of pituitary preparations, this method may
be used to measure the progesterone-like activity of other steroid com-
pounds in stimulating the secretion of mammogen.   Mixner and Turner
(1943) reported that 1 mg. of progesterone produced a unit response,
Pregneninolone was one-half, desoxycorticosterone acetate and dehydro-
androsterone one-third, acetoxypregnenolone one-sixteenth, and methyl
testosterone one twenty-fifth as effective as progesterone on a weight
basis.

This method may also be used to determine the effect of environ-
mental factors upon the secretion of mammogen.   For example, it
was shown that when progesterone was administered to assay animals
kept at high temperature (35°C.) the response was poor, whereas the
administration of pituitary preparations produced full response.

The influence of other hormones upon mammary gland growth may
also be investigated by this technic.   Thus the influence of thyroxine
or thiouracil may be studied.   In the case of progesterone, it was observed
that the simultaneous administration of thyroxine increased its activity
about 33.3%.

5. Comparison of Methods

In a personal communication, Trentin (1949) stated that the female
mouse method is the better of the two methods for pituitary materials,
giving a much more uniformly graded response curve because of the
basal estrogen injection.   The male mouse assay, although not giving
uniform response curves for pituitary material is much more satisfactory
when applied to the assay of estrogens.   In the assay of pituitary mate-
rials, the male mouse method might be used as a quicker and easier
means for qualitative screening or rough quantitative work, but the final
assays should be conducted by the female mouse method.

V. SUMMARY

That the anterior lobe of the hypophysis plays some role in the
growth of the mammary gland as well as in lactation is gaining wide

acceptance, but the question whether the gonadal hormones are primary or secondary in importance continues to be debated (Folley and Malpress, 1948). At the present time our observations lead us to believe that there is *one* pituitary mammogenic hormone of protein nature. It is secreted at a low level more or less constantly into the blood stream. The role of estrogen either systemically or by local application in physiological amounts is to increase the vascularity of the stromal tissue and the permeability of the capillaries so as to permit increased amounts of the circulating mammogen to bathe the growing mammary ducts.

In pregnant animals or those injected with progesterone or related compounds, the pituitary is stimulated to secrete increased amounts of mammogenic hormone. In the absence of circulating estrogen, the level of mammogen may be sufficiently high to stimulate duct and lobule-alveolar growth, but in the presence of estrogen the permeability of the capillaries is increased and very rapid mammary gland growth occurs. Progesterone secretion declines or is overridden physiologically during the latter part of pregnancy, and the secretion of mammogen declines, but the secretion of lactogen increases owing to the increasing secretion of estrogenic hormone.

The lactogenic hormone may possess some mammary gland stimulating properties but it is not believed to be identical with the mammogenic hormone which stimulates the growth of the lobule-alveolar system during pregnancy because this growth is not associated with lactation (Mixner and Turner, 1943). The role of the lactogenic hormone in addition to stimulating the secretion of milk may be to provide the stimulus to slow epithelial cell division sufficient to replace the losses of these cells during lactation. If further research with highly purified lactogenic hormone continues to show mammary growth-stimulating properties, it would meet the specifications of a pituitary mammogen as originally postulated, since its secretion is stimulated by estrogen (Meites and Turner, 1948).*

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CHAPTER XII

Growth Hormone

By FRANCIS S. GREENSPAN, C. H. LI, M. E. SIMPSON,
AND HERBERT M. EVANS

CONTENTS

I. INTRODUCTION

A review of methods for the bioassay of growth hormone has been limited in the past by the lack of a pure growth hormone preparation with which various assay methods could be standardized and compared. With the isolation and purification of pituitary growth hormone by Li *et al.* (1944, 1945) a standard preparation has become available. It is the purpose of this communication to review the methods suggested for the bioassay of growth hormone, to analyze the accuracy, sensitivity, and specificity of each, and to report some of the methods which have been standardized with the pure hormone.

Methods which have proved useful in the bioassay of growth hormone, and others which have been suggested as assay procedures are outlined in Table I. A direct comparison of these methods is difficult since they have been standardized in different laboratories, under different conditions, and with hormone preparations of different potency and purity. However, certain of the methods have been studied in the same laboratories with the same hormone preparation, and from these data some evaluation of the procedures can be attempted. The methods listed in Table I are divided into those which have been thoroughly studied and have been generally accepted assay procedures, and those

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which have been suggested for the bioassay of growth hormone but which either have not been accepted or have not been sufficiently explored.

TABLE I

Methods Available for the Bioassay of Growth Hormone

A. Well-Established Procedures:
1. Increase in weight of normal plateaued rats.
2. Increase in weight of hypophysectomized rats.
3. Increase in weight of dwarf mice.
4. Increase in tail length of hypophysectomized rats.
5. Increase in width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats.

B. Suggested Procedures:
1. Increase in liver weight.
2. Increase in weight in stilbestrol-treated rats.
3. Changes in the nitrogenous constituents of the blood.
4. Changes in the serum phosphorus or phosphatase.
5. Changes in the nitrogen or phosphorus balance.
6. Increase in protein synthesis demonstrable with radioactive tracers.

II. WELL-ESTABLISHED PROCEDURES FOR THE BIOASSAY OF GROWTH HORMONE

1. Increase in Weight of Normal Plateaued Rats

One of the first noted and most easily demonstrable effects of growth hormone is the acceleration of the growth of normal rats (Evans and Long, 1921; Evans, 1923; Evans *et al.*, 1948). It has been observed that the growth rate of the female rat diminishes rapidly after about 100 days of age, and soon a period of growth stasis, or a "plateau" in the growth curve is reached. This phenomenon has been utilized in what probably was the first satisfactory test for the bioassay of growth hormone, the increase in weight of the normal plateaued female rat. The details of the procedure as used in this laboratory have been outlined (Evans and Simpson, 1931; Evans *et al.*, 1933). Briefly, adult female rats of the Long-Evans strain, 5 to 6 months old, weighing 220 to 280 g., in which growth stasis can be demonstrated by failure to gain more than 10 g. in 20 days, are satisfactory for the assay procedure. Male animals have not been as satisfactory. The diet and the animal room conditions should be kept constant. The animals are weighed at 5-day intervals. Injections of hormone are made intraperitoneally daily for a specified period of time. Originally a 20-day test period was used, but a 15-day injection period is adequate (Marx, Simpson, and Evans, 1942). A "growth hormone unit," i.e., the minimum amount of hormone to produce a "significant" increment in body weight, was defined as that amount of growth hormone which when given under the conditions mentioned above

will produce an average body weight gain of 2 g. per day in a group of at least 6 animals. Shorter injection periods of 5 or 10 days, have been suggested by Light *et al.* (1940), and Chou *et al.* (1938), but in this laboratory these injection periods have proved too short for quantitative determinations. Fønss-Bech (1947) has used an injection period of 21 days.

According to standard methods for the evaluation of biological assays, the accuracy of a method for the bioassay of growth hormone can be analyzed by the ratio λ of the variance of the slope of the log-dose response line, the estimate of which is $\lambda = s/b$, where s is the standard deviation about the curve, and b is the slope of the curve (Bliss and Cattell, 1943). It has been instructive to apply this analysis to the data obtained by several authors with the plateaued rat weight test. These calculations are presented in Table II. From the data obtained by Marx *et al.* (1942) with groups of 30 animals at each dilution of hormone it can be determined that λ = 0.198, which represents a high degree of precision. From the data obtained by Fønss-Bech (1947) using a Wistar strain of rats, and an injection period of 21 days, in groups of 10 animals the index of precision λ = 0.226, which is satisfactory. It is apparent that the method has a fair degree of accuracy even in different laboratories.

TABLE II

The Accuracy of Several Methods for the Bioassay of Growth Hormone

Method	Duration injection period, days	S.D. (s)	Slope (b)	λ[a]	Author
Plateaued rat weight test	15	5.0	25.2	0.198	Marx *et al.*, 1942
	21	4.08	18.07	0.226	Fønss-Bech, 1947[b]
Hypophysectomized rat weight test	10	6.65	25.12	0.265	Marx *et al.*, 1942[c]
	7	4.95	14.0	0.354	Bülbring, 1938[c]
	14	4.18	10.40	0.402	Fønss-Bech, 1947
Dwarf mice weight test	14	0.78	3.34	0.234	Fønss-Bech, 1947[d]
	14	0.166	2.48	0.670	Fønss-Bech, 1947[b]
Tail length test	7	0.158	2.97	0.532	Fønss-Bech, 1947[b]
	14	1.19	5.30	0.225	Dingemanse *et al.*, 1948
Tibia test	4	24.6	79.4	0.310	Greenspan *et al.*, 1949

[a] Estimate of λ (see text).
[b] As corrected by Gjeddebaek, 1948.
[c] Calculated by Bliss and Cattell, 1943.
[d] Selected data.

The sensitivity of a method for bioassay can be described in terms of the minimum amount of hormone to produce a significant response under stated conditions. With the plateaued rat weight test, these conditions have already been defined above as the "growth hormone unit." It is impossible to compare the sensitivity of one method to another unless both have been standardized with the same preparation. Fortunately, there have been some studies in which the same preparation has been used in the standardization of several bioassay methods. In this laboratory, for example, a single "globulin fraction" rich in growth hormone was used to standardize the plateaued rat weight test, the

TABLE III

Comparison of the Sensitivity of Various Methods of Growth Hormone Bioassay Standardized with the Same Hormone Preparation

Growth hormone preparation	Bioassay method	Threshold response		
		Definition	Total dose growth hormone	Reference
Globulin fraction	Pleateaued rat weight test	Weight gain 2 g. /day for 15 days	15.000 mg.	Marx *et al.*, 1942
	Hypophysectomized rat weight test	Weight gain 1 g. /day for 15 days	0 520 mg.	Evans *et al.*, 1943
	Tibia test	Increased width epiphyseal cartilage 40 μ	0.048 mg.	Evans *et al.*, 1943
Pure growth hormone	Hypophysectomized rat weight test	Weight gain 1 g. /day for 10 days	0.090 mg.	Li *et al.*, 1945
	Tibia Test	Increased width epiphyseal cartilage 40 μ	0.005 mg.	Greenspan *et al.*, 1949
Partially purified growth hormone	Pleateaued rat weight test	Weight gain 2 g. /day for 21 days	34.000 mg.[a]	Fønss-Bech, 1947
	Hypophysectomized rat weight test	Weight gain 1 g. /day for 14 days	12.000 mg.	Fønss-Bech, 1947
	Tail length test	Increase in tail length 6 mm. in 7 days	14.000 mg.[a]	Fønss-Bech, 1947
	Dwarf mice test	Increase in weight of 50 % in 14 days	6.000 mg.	Fønss-Bech, 1947

[a] The total amount administered did not achieve a threshold response.

hypophysectomized rat weight test and the tibia test (*vide infra*). In the pleateaued rat weight test, 15 mg. of this preparation represented a minimal effective dose, whereas in the hypophysectomized rat weight test and the tibia test, 0.5 mg. and 0.05 mg. respectively were necessary for a minimal effective dose. A similar result was obtained independently by Fønss-Bech (1947) (Table III). It is apparent that the plateaued rat weight test requires a considerable quantity of hormone in order to

obtain a satisfactory response. From the data cited in Table III (globulin fraction) it can be calculated that the hypophysectomized rat test is about 30 times as sensitive, and the tibia test about 300 times as sensitive as the plateaued rat weight test.

The specificity of the plateaued rat weight test must be considered in terms of the factors controlling the growth of the animal. These would include diet, environmental conditions, and the interaction of a number of hormones. On the other hand, if the above conditions are maintained, growth hormone is the only substance which will cause rapid and continuous growth of the animal. Among the hormonal substances which will cause small weight increases in the normal animal are testosterone, lactogenic hormone, and thyroxin. In addition, there is the problem of synergism of other hormones with growth hormone, viz., thyroxine plus growth hormone will produce a larger increment in growth than growth hormone alone (Smith, 1933; Evans *et al.*, 1939). Adrenocorticotrophic hormone will antagonize some of the growth effects of growth hormone (Evans, Simpson and Li, 1943; Li and Evans, 1947). It is important, therefore, in the bioassay of growth hormone fractions, to determine the degree of contamination by other hormones in order to assess the possibility of synergism or antagonism in the reaction. It should be pointed out, however, that growth hormone preparations of different potency tested by this method have produced dose-response curves which are very nearly parallel (Marx *et al.*, 1942), indicating that the response is reproducible. This is well illustrated in the assay data obtained in this laboratory with a partially purified growth hormone preparation (globulin fraction, Marx *et al.*, 1942) and with pure growth hormone (Evans *et al.*, 1949) which are presented graphically in Fig. 1. The assays were run on the same Long-Evans strain of rats and under similar conditions, but 7 years apart, and the slopes are very nearly parallel. There is some variation in the responsiveness of the animals at different times of the year, so that it is essential to control assays with a standard hormone preparation, preferably the pure hormone, whenever possible.

In summary, the advantages of the plateaued rat weight method for the bioassay of growth hormone are these: the animals require no operative procedure and are easily maintained; the animals tolerate injections of impure material; the test procedure is simple, and it has a high degree of accuracy. The limitations of the method are that it is relatively insensitive and large amounts of hormone are necessary to produce a satisfactory response. In addition, the animals must be rigorously standardized, the assay must be controlled with a standard reference substance, and the possibilities of synergistic and antagonistic reactions of other hormones must be considered when impure preparations are assayed.
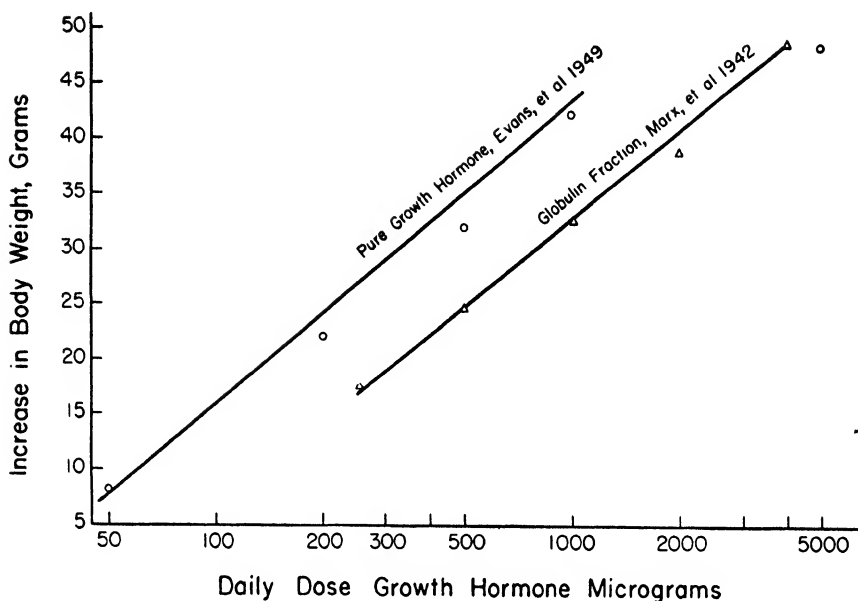
FIG. 1. Response of normal plateaued rats to partially purified growth hormone and to pure growth hormone, 15 day test.

2. Increase in Weight of Hypophysectomized Rats

Cessation of growth in the hypophysectomized rat and its resumption by implantation of whole pituitary glands was first demonstrated by Smith (1926, 1927, 1930). Among the first to utilize these phenomena in the assay of growth-promoting extracts of the anterior hypophysis were Van Dyke and Wallen-Lawrence (1930), Thompson and Gaiser (1932), Evans *et al.* (1933), and Van Dyke (1936). These workers used a short assay period of 4 to 5 days as a qualitative rather than a quantitative procedure. Collip *et al.* (1933) used rats 12 to 14 days postoperative and a treatment period of 15 days. Evans *et al.* (1938) first standardized the procedure using 2- to 3-month-old female rats, 20 to 30 days postoperative, with a treatment period of 10 days. Under these conditions a growth hormone unit was defined as that amount of hormone necessary to produce an increase in the weight of the hypophysectomized rat of 10 g. in 10 days. Later immature female rats were used and the postoperative period shortened to 6 to 10 days (Fraenkel-Conrat *et al.*, 1940). There has been some disagreement on the duration of the injection period. Evans *et al.* (1938), Chou *et al.* (1938), and Abraham (1942) used a 10-day period, while Bülbring (1938) and Freud (1938) considered 7 days sufficient. Marx, Simpson, and Evans (1942) studied the dose-

response curves for injection periods from 5 to 20 days and demonstrated that 10 to 15 days was most satisfactory.

The procedure for the hypophysectomized rat weight test, was used in this laboratory has been presented by Marx, Simpson, and Evans, 1942. Briefly, it may be summarized as follows: immature female rats are hypophysectomized at 26 to 28 days of age and, if evidence for completeness of operation has been obtained, are used for assay 10 to 12 days later. The criteria for completeness of hypophysectomy are: limitation of body weight gain to 7 g. in the preinjection period, impairment of body tonus, maintenance of infantile hair, and the final examination of the sella turcica at autopsy. The hormone is injected intraperitoneally daily for 15 days, or, more recently for 10 days (Li, Evans, and Simpson, 1945). An hypophysectomized rat weight unit has been defined as the amount of growth hormone that produces, under the above conditions, an average body weight gain of 1 g. per day in groups of 6 to 10 animals, for the number of days specified.

The accuracy of this method is indicated by the calculations of λ in Table II. It will be noted that the hypophysectomized rat weight test is slightly less precise than the plateaued rat weight test. This is substantiated by the fact that with the same hormone preparation a larger multiple of the dose is necessary to produce a significant increment of response with the hypophysectomized rat weight test than with the latter method (Marx, Simpson, and Evans, 1942). On the other hand, the hypophysectomized rat is considerably more sensitive in its response to growth hormone than the normal plateaued rat. In terms of the growth hormone unit it has already been pointed out that this test is about 30 times as sensitive as the normal plateaued rat test (Table III). Greater accuracy can be obtained in the hypophysectomized rat weight test if a longer injection period of 15 to 20 days is used.

In regard to the specificity of the hypophysectomized rat weight test, the comments made in the previous section regarding the synergism and antagonism of several hormones with growth hormone hold true for the hypophysectomized as well as the normal animal (Marx *et al.*, 1943). It is of interest, however, that a partially purified growth hormone preparation and pure growth hormone, tested in the hypophysectomized animal several years apart, yield standard curves of nearly parallel slope (Fig. 2).

The hypophysectomized animal is considerably more delicate than the normal animal and will not tolerate toxic extracts. As with the previous method it is important to standardize the animals in regard to age, weight, sex, etc. Several workers have pointed out the inadvisability of using the same animals for more than one assay inasmuch as the

response to the second assay series will be less than to the first (Chou et al., 1938; Bülbring, 1938; Marx, Simpson, and Evans, 1942).

In summary, the main advantage of the hypophysectomized rat weight test is the increased sensitivity of the assay method. The accuracy and specificity are about the same as that of the normal plateaued rat. The limitations of the method include the necessity for a careful operative procedure, the delicacy of the test animal, and the extreme importance of a rigorous standardization of the animals. As with the previous method, a standard reference substance should be
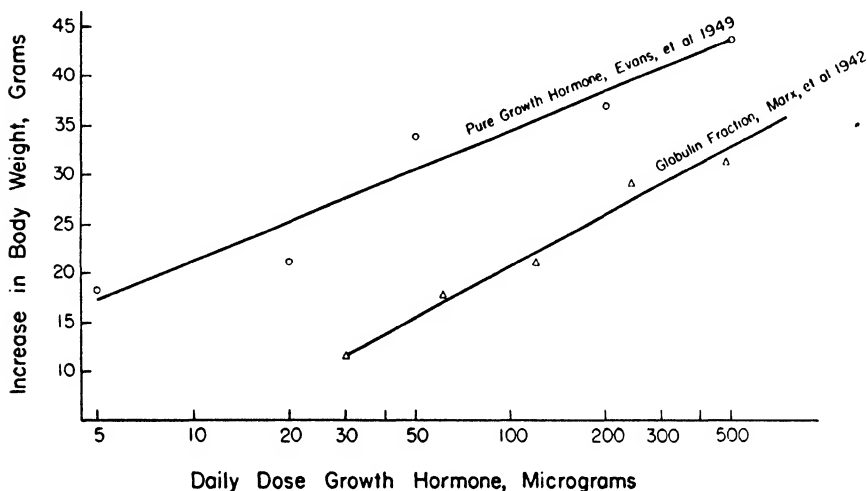
FIG. 2.   Response of hypophysectomized rats to partially purified growth hormone and to pure growth hormone, 15 day test.

used to control each assay and the possibilities of synergism and antagonism of other substances in impure preparations must be considered.

3. Increase in Weight of Dwarf Mice

A strain of mice with hereditary dwarfism associated with a deficiency in the eosinophiles of the anterior hypophysis was described by Snell in 1929. Smith and McDowell (1930, 1931) demonstrated that pituitary gland implants restored normal growth to these animals, and Kemp (1933, 1934) produced growth with a crude pituitary extract containing growth hormone. Dodds and Noble (1936) suggested this as a method of standardization of growth hormone, although Kemp and Marx (1937) noted that crude prolactin, thyrotrophic hormone, and thyroxin also restored normal growth in the dwarf mouse. A procedure for bioassay

of growth hormone using the dwarf mouse has been standardized by Kemp (1946) and has been studied in detail by Fønss-Bech (1947).

The details of the bioassay procedure, as used by Fønss-Bech (1947), are as follows. Groups of 10 mice each, of both sexes, age 6 to 8 weeks and weighing 4.5 to 7 g. are maintained with their mothers at a constant temperature of 21°C. Sterile extracts are injected subcutaneously daily except Sunday, and the animals weighed on a torsion balance twice weekly. A dwarf mouse unit is defined as the amount of growth hormone which when administered under the above conditions for 2 weeks effects an average weight increase of 50% of the initial weight in 10 animals. Kemp (1946, 1948) considers that an injection period of 3 weeks is essential for accuracy.

The accuracy of the dwarf mice bioassay method has been studied by Fønss-Bech (1947) and discussed by Gjeddebaek (1948). The calculation of λ for all the data on the 2-week method is 0.670, and for selected data 0.234. For the 3-week test, $\lambda = 0.228$ (Kemp, 1948). Thus the accuracy of this procedure is relatively poor for a 2-week test but improves with a 3-week injection period (Table II). An index to the sensitivity of the method can be gained by comparison of the minimal effective dose of the same hormone preparation tested by this and other methods. This information can be obtained from data presented by Fønss-Bech (1947) and is summarized in Table III. It will be noted that of the 4 methods tested by Fønss-Bech, the dwarf mouse test was the most sensitive.

In regard to specificity, the problem of synergism and antagonism of various hormones exists in this procedure as well as in the previously described procedures. The growth-promoting effects of thyroid and thyrotropic hormone and their synergistic effect with growth hormone have been described by Kemp and Marx (1937), Evans *et al.* (1939), and Laqueur *et al.* (1941). A synergism between crude prolactin and thyrotropic hormone in their effect on dwarf mice was described by Bates *et al.* (1935).

The limitations of the method are primarily the difficulties in maintaining the strain of animals over long periods of time and the delicacy of the animals. In addition, the order of accuracy of the method is low with short term injection periods. It has the obvious advantage that the need for an operative procedure is eliminated.

4. Increase in Tail Length of Hypophysectomized Rats

This method for the bioassay of growth hormone was proposed and standardized by Freud (1938), Freud *et al.* (1939), Freud and Dingemanse

(1946) and has been recently reviewed by Dingemanse *et al.* (1948). The details of the procedure as noted in the latter report are as follows. Male rats age 6 to 8 weeks and weighing about 100 g. are hypophysectomized. Groups of 5 animals were injected daily intraperitoneally or subcutaneously, beginning the day of or the day after hypophysectomy, and continuing for 1 or 2 weeks. Pre- and post-treatment caudal skiagrams are taken, and the tail length, i.e., the sum of 27 vertebrae, intervertebral spaces, and skin tip, is carefully measured. A threshold response or a growth hormone unit is defined as the amount of growth hormone which, given under the conditions above, produced an average increment in tail length of 6 mm. in a 7-day test, or 9 mm. in a 14-day test. A daily dose of 0.35 mg. of an acetone dried powder was equivalent to one growth hormone unit. These authors have not considered a dose-response curve to be of value in the assay of growth hormone specimens, but have compared all unknown specimens with the single standard powder in terms of the equivalent amount necessary to produce a threshold response.

From the data presented by Dingemanse *et al.* (1948) on 806 animals it can easily be demonstrated that a straight line relationship exists between the response of the tail length and the logarithm of the daily dose. The slope of the line for the 2-week assay data was calculated: $b = 5.32$, and mean standard error $s = 1.19$, whence $\lambda = 0.225$, indicating a fair degree of accuracy for the procedure (Table II). These calculations are based on large groups of animals (43 to 110). Fønss-Bech (1947) using groups of 10 hypophysectomized female animals, injected for 7 days, also obtained a satisfactory dose-response curve. From his data it was calculated that $\lambda = 0.532$ (Gjeddebaek, 1948), indicating a considerably lesser degree of accuracy.

An indication of the sensitivity of this method is obtained from the comparative study of Fønss-Bech (1947) (Table III). With a single growth hormone preparation it was found that a total dose of 14 mg. did not produce a threshold response as defined by Freud *et al.* (1939), whereas the same amount of hormone administered to hypophysectomized rats for 2 weeks (weight test) produced a greater than threshold response as defined by Marx, Simpson, and Evans (1942). Only 6 mg. of this same preparation was necessary to produce a threshold response by the dwarf mouse assay method. It may be said, therefore, that the sensitivity of this method is less than that of the hypophysectomized rat weight method and considerably less than the dwarf mouse method. With regard to specificity, it is likely that the same factors that have already been discussed, namely, thyroid and thyrotropic hormone, may have a synergistic action and other substances may antagonize the action

of growth hormone on the growth of the tail. These factors will be discussed in more detail in relation to the tibia test (*vide infra*).

The proponents of this test have maintained that a skeletal indicator of growth, such as the increase in the length of the tail of the hypophysectomized animal, is a more decisive indicator of the growth principle than increment of weight. We do not propose to enter into a discussion of this problem here except to comment that it is apparent that a direct log dose-response relationship exists for both increments of skeletal length and body weight, and under certain conditions either may be useful as a bioassay procedure. The tail length assay is limited by the necessity for hypophysectomy, the insensitivity of the method, and the duration of the injection period (2 weeks) necessary for satisfactory results.

5. The Tibia Test

The cessation of growth of the epiphysis following hypophysectomy in the dog and cat was first reported by Dott and Fraser (1923). Handelsman and Gordon (1930) obtained evidence that alkaline extracts of the anterior pituitary stimulated growth of bone by feeding rats madder (a root containing alazarin and purpureum dye) and observing the increased color of the newly formed bone. They proposed this as an assay procedure commenting that " . . . in rats weighing 190 to 215 grams the potency of an extract can be evaluated in as short a time as two weeks." Lucke and Hückel (1933) and Silberberg (1935) used acid extracts of the hypophysis in rats and guinea pigs respectively and noted stimulation of epiphyseal cartilage plates. Freud *et al.* (1939) considered the primary point of action of growth hormone to be on cartilage and coined the term "chondrotrophic hormone." Ross and McLean (1940) noted that preparations of the anterior pituitary caused resumption of growth and widening of the epiphyseal plate in 6-month-old plateaued rats, both male and female. A careful study of the effect of pituitary growth hormone on the epiphyseal disk of the tibia of the normal and the hypophysectomized rat, in relation to the age of the animal, was made by Ray *et al.* (1941). It was demonstrated that hypophysectomy was followed by a marked reduction in the width of the epiphyseal cartilage, reflecting the disturbance of the equilibrium that exists between chondrogenesis and osteogenesis. Furthermore, administration of growth hormone to the hypophysectomized animal brought about a marked increase in the width of the cartilage plate, most pronounced in young animals by stimulating first chondrogenesis and then osteogenesis, until the equilibrium is re-established. It was later shown by Kibrick *et al.* (1941) that the epiphyseal cartilage response in young hypophysectomized rats

to injections of increasing amounts of growth hormone over a 4-day period fell into a straight line dose-response curve when plotted on a semilogarithmic scale.

On the basis of these findings, the tibia test for the bioassay of pituitary growth hormone was proposed by Evans, Simpson, Marx, and Kibrick in 1943. The test has recently been restandardized with pure growth hormone by Greenspan *et al.* (1949). The procedure, in brief, is as follows. Immature female rats maintained under standardized conditions are hypophysectomized at 26 to 28 days. The criteria for
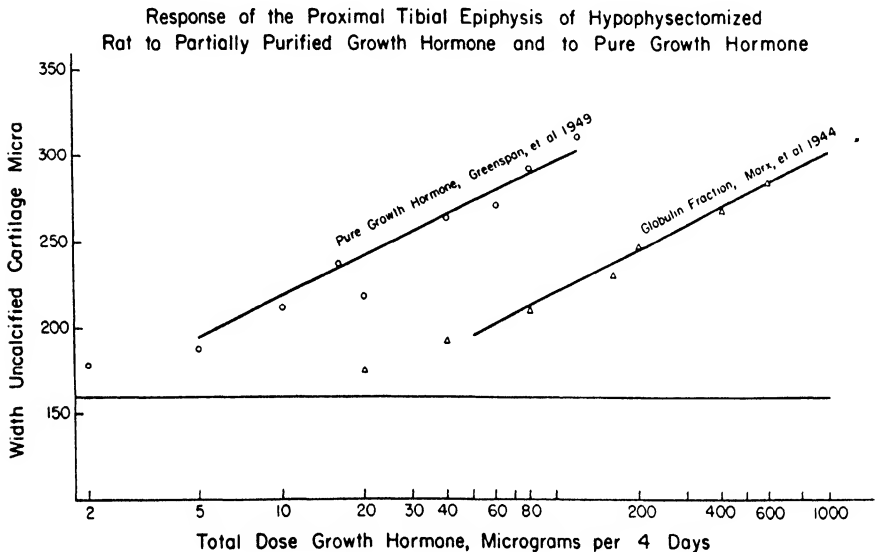
FIG. 3.   Response of the proximal tibial epiphysis of hypophysectomized rat to partially purified growth hormone and to pure growth hormone.

the completeness of hypophysectomy have already been mentioned. After a postoperative interval of 12 to 14 days, the animals receive daily intraperitoneal injections of growth hormone for 4 days. Twenty-four hours after the last injection, the animals are sacrificed with ether or chloroform, one or both tibiae are dissected free from soft tissue, and split with a sharp razor in the midsagittal plane. The bone halves are stained immediately or fixed in 10% neutral formalin. Prior to staining, the bone halves are washed in water for ½ hr., immersed in acetone at least 1 hr. and then washed in water again for ½ hr. They are then placed in freshly prepared 2% silver nitrate for 1½–2 min., rinsed once in water, and while under water exposed to a strong light until the calcified portions appear dark brown. They are then immersed in 10%

sodium thiosulfate for 25 to 30 sec. and washed in running water for ½ hr. They are stored in 80% ethanol in the dark. The width of the uncalcified epiphyseal cartilage is measured under the low power of the microscope, using a calibrated micrometer eyepiece. A minimum of 8 to 10 readings are taken and the results averaged.

An analysis of the method has been made by Greenspan et al. (1949). It was found that with groups of 8 to 10 animals, between dosage levels of 5 to 120 μg. of pure growth hormone per animal per 4 days, a straight line log dose-response curve was obtained (Fig. 3). Over this range, approximately doubling the dose of hormone produced a significant increment in the response. It is calculated that λ = 0.301, indicating a fair degree of accuracy for the method (Table II). The great advantage of the method is its high sensitivity. It was demonstrated that a total dose of 5 μg. of pure growth hormone was sufficient to produce a significant increase in the width of the cartilage plate in the hypophysectomized rat under the above conditions. This is about one-tenth to one-twentieth the quantity of pure hormone necessary to produce a significant increment in body weight of the hypophysectomized rat in the 10-day test (Table 3). A second advantage of the procedure is the short injection period of 4 days. It has been shown that shorter injection periods are unsatisfactory, and that increasing the injection period does not add to the sensitivity of the response (Greenspan et al., 1949). The specificity of the reaction has been studied by Simpson et al. (1944), Marx et al. (1944), Becks et al. (1946), and reviewed by Greenspan et al. (1949). In general, it has been found that a number of substances, such as thyroxin, thyrotropic hormone, lactogenic hormone, and testosterone, may cause an increase in the width of the epiphyseal plate of the hypophysectomized rat from a control level of about 155 μ to a range of 175 to 185 μ, but increasing the dose of these substances does not increase the width above this "non-specific" response range. The problem of the synergistic effect of thyroid or thyrotropic hormone plus growth hormone and of the antagonistic effect of adrenocorticotropic hormone has been discussed in the above reports and emphasizes the importance of simultaneous estimations of the degree of contamination by other hormones of the preparation to be tested. It is important to point out that a partially purified specimen of growth hormone standardized by this method in 1943 and the pure hormone standardized in 1949 yielded log dose-response curves closely parallel, indicating the reproducibility of the results of the tibia test (Fig. 3). The limitations of the method include the omnipresent need for careful standardization of the animals and the conditions of the assay, the operative procedure, and the delicacy of the hypophysectomized animals. These limitations are offset by the

greatly increased sensitivity of the method, the short injection period, and the reproducibility of the assay results.

III. Suggested Methods for the Bioassay of Growth Hormone

Some of the methods which have been suggested for the bioassay of growth hormone but which have not been generally accepted are listed in Table I.   Increase in liver weight in animals treated with growth hormone was suggested as an assay procedure by the work of Lee and Freeman (1940), but Fraenkel-Conrat *et al.* (1942) demonstrated that thyroxin and thyrotropic hormone caused a much more marked increase in liver weight than did purified growth hormone, implying that the effects noted by Lee and Freeman may have been due in part to impurities in the hormone preparation used.   Griffiths and Young (1942) suggested the use of stilbestrol-treated male rats for the bioassay of growth hormone.. These animals cease to gain weight, although skeletal growth continues. On treatment with growth hormone, weight gain is resumed, and the increase in weight over a period of 14 days of treatment can be expressed in a straight line log dose-response curve.   The authors did not consider the method entirely satisfactory because of the dissociation between weight stoppage and skeletal growth in the estrogen treated animal.

Teel and Watkins (1929), Teel and Cushing (1930), Gaebler (1933), and others have reported a depression in the non-protein nitrogen, urea, and amino acids of the blood of dogs after treatment with anterior pituitary extracts.   Goss and Gregory (1935) demonstrated a 55% fall in the concentration of glutathione in the liver of the rat 12 hr. after a single injection of an hypophyseal extract.   These methods were reviewed by Lee (1938) who found that none were suitable for bioassay purposes. Li *et al.* (1947) have reported the rise in serum alkaline phosphatase in hypophysectomized rats after the administration of growth hormone, and Li *et al.* (1949) have noted the rise in the serum phosphorus in the hypophysectomized rat after growth hormone treatment, but a satisfactory log dose-response curve has not been obtained with these procedures.   Since growth hormone is a protein anabolic hormone, it would seem that nitrogen storage or phosphate retention might be satisfactory criteria for the assay of the hormone.   The storage of nitrogen and phosphorus under treatment with crude anterior pituitary extracts was demonstrated in dogs by Gaebler and Price (1937), Gaebler and Robinson (1942), and more recently, the effects of pure growth hormone on nitrogen storage have been reported by Marx *et al.* (1942), Gordan *et al.* (1948) and Whitney *et al.* (1948).   Bartlett and Gaebler (1948) have suggested a qualitative procedure for the assay of growth hormone based upon nitrogen storage in dogs, in which preparations are " . . . con-

sidered satisfactory if, when injected subcutaneously in a large single dose of 200 mg., they produce nitrogen storage of 0.5 grams per kg. in dogs maintained on a complete diet high in protein." This is a nice demonstration of the protein anabolic nature of the hormone, but is, at best, a qualitative procedure. Friedberg and Greenberg (1947) have reported that the incorporation of sulfur from methionine labeled with S^{35} into the protein of skeletal muscle is increased about 70% by the administration of growth hormone. This technic offers interesting possibilities. In general, the biochemical procedures outlined above, because of lack of sensitivity, specificity, or failure to fall into a satisfactory log dose-response curve, have not proved practical for the bioassay of growth hormone.

IV. COMMENT ON METHODS

A word of comment should be made concerning the manner in which bioassay methods may be used. A given reaction, whether it be the gain in weight of a normal, hypophysectomized or dwarf rat, the growth of the tail, or the tibia test, may be utilized in one of two ways. Either a log dose-response curve is obtained from a standard preparation and unknown preparations evaluated against the standard curve (the location of the curve must, of course, be checked with the standard preparation), or a threshold response is determined and the unknown compared directly with the standard in terms of the amount of each necessary to produce the desired response. Most workers are agreed that when a dose-response curve falls into a straight line on a semilogarithmic scale it is advantageous to utilize the curve because of the ease of determining the limits of the assay procedure, the statistical significance of given increments in the response, and the accuracy of comparative determinations. The methods with which we are most familiar in this laboratory are the plateaued rat weight test, the hypophysectomized rat weight test and the tibia test. Each of these reactions, between certain limits, falls into the desirable log dose-response curve. Thus comparisons of the sensitivity and accuracy of the methods can be made. It is to be emphasized that in each of these methods, although the responsiveness of the animals may vary from time to time, the slope of the curve remains relatively constant, so that a single standard point is usually all that is necessary to locate the curve. This allows a considerable conservation of effort and material. It would obviously require much more hormone and many more animals if dilutions of the unknown and solutions of the standard had to be determined at each assay in order to locate the equivalence of the threshold point.

We have found that pure growth hormone, as prepared by the method

of Li *et al.* (1945), has been an extremely satisfactory standard preparation. The dry powder is stable indefinitely at room temperature in a vacuum desiccator and recent batches of pure hormone are wholly comparable to the initial ones. It is to be recommended that such a preparation be adopted as a standard preparation against which other preparations may be compared.

The problem of the "most satisfactory" assay procedure for growth hormone is a difficult one. Each of the procedures described has its advantages and limitations, its proponents and opponents. In general we have found that the tibia test is the most sensitive index of growth hormone activity that we have used. On the other hand, the plateaued rat weight test, and the hypophysectomized rat weight test are equally as accurate when larger amounts of hormone are available. Each of the tests is characterized by a relative lack of specificity, but on the other hand, in each procedure the slopes of the log dose-response curves are quite reproducible with the pure hormone. Finally, each procedure requires that the test animals be rigorously standardized and the conditions of the assay be reproduced with meticulous constancy if the results are to be at all comparable.

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Androgens

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CONTENTS

I. INTRODUCTION

Androgens by definition are substances which possess characteristic biological activity affecting the secondary sex characters of various male

animals. Traditionally androgens have been assayed by the comb response in the fowl, and on the seminal vesicles and prostate of the rodent. It is the purpose of this section to deal with the technics employed, their sensitivity, and their reproducibility.

Androgens as a class possess a variety of biological activities which may be designated as non-sexual, such as influences on various endocrine and non-endocrine glands, nitrogen metabolism, muscle mass, electrolyte metabolism, and specific enzyme concentrations of various glands and tissues. These actions have not been used in the biological assay of androgens, and indeed by definition they could not be employed until shown to be correlated with the development of the sex characters. The assay of androgens using these non-sexual end points is not considered in this chapter.

II. Surgical Procedures

Since the methods to be described are concerned primarily with capons and castrated rats, the surgical procedures are given below.

1. Caponizing

White or Brown Leghorn cockerels are usually employed because of their relatively high sensitivity to androgens and because they are usually readily available. The cockerels are operated at approximately 6 weeks of age, but with care the operation can be conveniently done as early as 1 to 2 weeks of age. The 6-week-old animals are fasted for 24 hr. before surgery, while 1- to 2-week-old cockerels need be fasted for only 6 to 8 hr.

After fasting, the animals are anesthetized with ether and placed on their sides. The incision is made between the last 2 ribs, the muscle layer divided, and the incision pulled apart with small retractors. The testis is found close to the midline of the posterior abdominal wall, alongside the vena cava. The capsule enclosing the testis is cut and the gonad removed. It is imperative to remove the testis intact as fragments left behind usually are vascularized and persist, giving rise to incompletely caponized animals. The incision is closed by sewing. The second testis is removed in a similar fashion on the other side.

Even if great care is taken to remove the testis, some animals will show comb growth subsequent to operation. These animals, called slips, are not suitable for assay purposes and must be discarded.

2. Castration in the Rat

Under ether anesthesia, an incision is made in the tip of the scrotum large enough to permit the removal of the testis. A single ligature is

placed around the internal spermatic vessels, the deferential vessels, and the ductus deference. The testis and epididymis are removed. The incision is closed by a suture or by means of a wound clip (Griffith and Farris, 1942).

III. BIRD METHODS

1. Capon Comb Growth

Most of the workers using the capon comb test have employed the White or Brown Leghorn capon. The English game bantam has been found to be a relatively reactive breed, but the heavier breeds are reported to be less reactive. The Plymouth Rock capon has been shown to be one-fifth as sensitive as the Brown Leghorn (Callow and Parkes, 1935). The relative sensitivity of various breeds will be discussed further under comments on the use of the chick's comb for androgen assay (see p. 302).

A. INJECTION

i. *Method of Gallagher and Koch (1935)*. The method consists in determining the growth of the capon's comb after 5 daily intramuscular injections of the unknown and in comparing the comb response with that found for a standard preparation of crystalline material under the same experimental conditions.

This bioassay method may be employed in 1 of 2 different ways. The first consists essentially in using the original design of Gallagher and Koch (1935). In working with most urines the preferred standard is androsterone, since the greater part of the activity in human urines is due to this androgen. An exception to this is the urine from subjects with adrenal cortical tumors, where dehydroisoandrosterone makes up the bulk of the active androgens. In the original work of Gallagher and Koch (1935), the standard employed was a highly purified bull testis preparation, and a "characteristic curve" was determined which used the dose-response relationship.

Another design consists in running two concentrations of unknown in parallel with two concentrations of the standard, according to the design of Bliss (1944). This method is illustrated on p. 307.

At the beginning of an assay the sum of the length plus height ($L + H$) of each individual comb is determined by measurement with a millimeter rule placed directly on the comb. It is often of value to record the exact barble used for the determination of the height. The capons are injected intramuscularly, daily, for 5 consecutive days. The daily dose is contained in 1 ml. of olive or corn oil. Twenty-four hours after the last injection the combs are remeasured and the growth of the comb

expressed as the sum $(L + H)$ in millimeters. If the first method is used, the mean $L + H$ increment of 8 capons is referred to the standard curve and the unitage in international units (I.U.) read directly from the dose-response curve. If the second method is used, the relative potency of unknown and standard is calculated according to the method of Bliss (1944). Factors which must be considered are as follows.

1. The initial comb size is of importance; for every millimeter difference in the initial length (from 57 mm.) of the comb, a correction of 0.17 mm. of comb growth may be used.

2. The body weight of capons has only an insignificant effect on growth of comb.

3. The unknown and standard should be run under identical light conditions.

4. Before a capon is used again for an assay it is usually necessary to wait about one month to allow sufficient time for regression of the comb.

Gallagher and Koch (1935) found a mean error of 22.6% when the unknown was run in parallel with a standard, and groups of 16 to 25 capons were used for both the unknown and standard.

ii. *Method of Greenwood et al. (1935).* Greenwood *et al.* (1935) have employed the Brown Leghorn capon for the assay of androgens in a manner similar to that of Gallagher and Koch (1935). At the beginning of the experiment the length and height of the combs are measured with a millimeter rule. The hormone, in 0.2 ml. of oil, is injected once daily into the pectoral muscle. At the end of 3 or 5 days, depending on the individual experiment, the combs are remeasured and the response is determined as the difference in length and height of the comb between the pre- and post-injection measurements.

Table I and Figs. 1 and 2 illustrate the comb response after the 3- and 5-day injection periods, respectively. When the data of Greenwood

TABLE I

The Response of the Capon's Comb to Androsterone (Data of Greenwood et al., 1935)

Type of test days	Total dose mg.	Number of capons	Comb growth $L + H$ mm. $\pm$ S.E.[a]
3	0.3	5	0.6 $\pm$ 0.59
	0.6	5	1.8 $\pm$ 0.20
	1.2	5	5.4 $\pm$ 0.75
	2.4	5	7.8 $\pm$ 1.15
	4.8	5	9.8 $\pm$ 0.73
5	0.5	5	2.8 $\pm$ 1.34
	1.0	5	6.2 $\pm$ 0.85
	2.0	5	10.6 $\pm$ 1.03
	4.0	5	15.4 $\pm$ 1.11
	8.0	5	17.4 $\pm$ 0.80

[a] S.E. is the standard error of the mean.

et al. (1935) are plotted as the logarithm of the dose versus response, a linear function was realized for both the 3- and 5-day tests. This being the case, the slopes were calculated and the index of precision (λ) determined. For the 3-day test the slope (b) was 8.09 and λ was equal to 0.189, while for the 5-day test $b = 12.72$, and $\lambda = 0.180$.
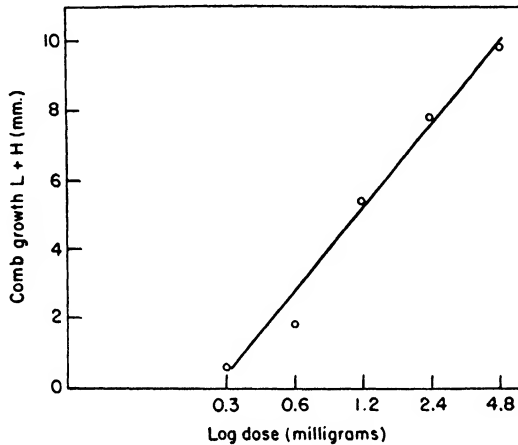
FIG. 1.   Capon's comb response to androsterone injected daily for 3 days (Greenwood *et al.*, 1935).
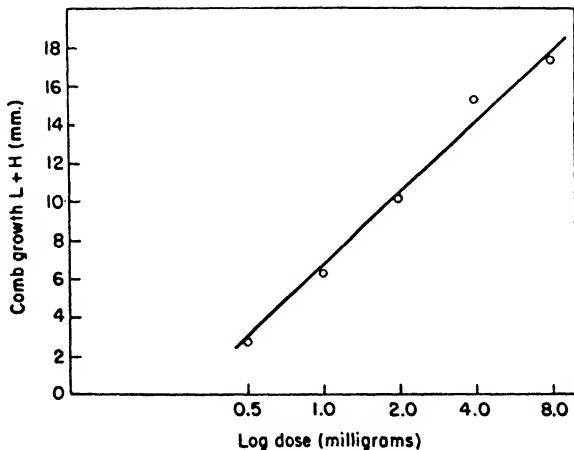
FIG. 2.   Capon's comb response to androsterone injected daily for 5 days (Greenwood *et al.*, 1935).

iii. *Method of Emmens* (*1939*).   Emmens (1939) has studied the capon's comb growth response to androsterone in the Brown Leghorn. The method is similar to that of Gallagher and Koch (1935). Brown Leghorn cockerels are caponized at 6 weeks of age and used at 6 to 9

months of age. The hormone is administered daily in 0.1 ml. of oil for 5 consecutive days. Comb measurements are done before treatment and 24 hr. after the last injection by the method already described (see p. 293).

Tables II and III present the data of Emmens (1939) concerning the response of the comb to androsterone after 3 and 5 days of injections,

TABLE II

The Response of the Capon's Comb to Androsterone by Injection (Data of Emmens, 1939)
Three Injections

Total dose I.U.	Number of capons	Mean comb growth $L + H$ mm. $\pm$ S.E.
0.75	10	1.70 $\pm$ 0.23
1.50	10	2.75 $\pm$ 0.23
3.00	8	3.25 $\pm$ 0.43
4.50	9	4.11 $\pm$ 0.41
6.00	9	5.11 $\pm$ 0.33
7.50	10	6.05 $\pm$ 0.57
9.00	9	7.33 $\pm$ 0.46

TABLE III

The Response of the Capon's Comb to Androsterone by Injection (Data of Emmens, 1939)
Five Injections

Total dose I.U.	Number of capons	Mean comb growth $L + H$ mm. $\pm$ S.E.
1.25	10	3.15 $\pm$ 0.32
2.50	10	4.50 $\pm$ 0.36
5.00	8	5.81 $\pm$ 0.43
7.50	9	7.77 $\pm$ 0.63
10.00	9	9.44 $\pm$ 0.47

respectively. The data at 3 or 5 days do not fit a linear relationship when the response is plotted against the logarithm of the dose (Fig. 3), but when the data are plotted as logarithm of dose versus logarithm of response, a linear relationship is found (Fig. 4). Making use of this latter relationship, the values of the slope have been calculated and the index of precision λ determined. In the 3-day test the slope was 0.568 and $\lambda = 0.197$, while in the 5-day test, the slope was 0.404 and $\lambda = 0.280$.

Table IV illustrates the estimated errors as calculated by Emmens (1939). Both the 3- and 5-day tests are considered.

iv. *Method of McCullagh and Cuyler (1939)*. The method is similar to that of Gallagher and Koch (1935). The pooled results are represented in Table V and Fig. 5. The total dose of androsterone ranges from 50

to 5000 μg. administered over a 5-day period. Between 200 and 5000 μg., a linear relationship of the log dose to response was found. From these data a slope of 10.52 and $\lambda = 0.271$ was found.
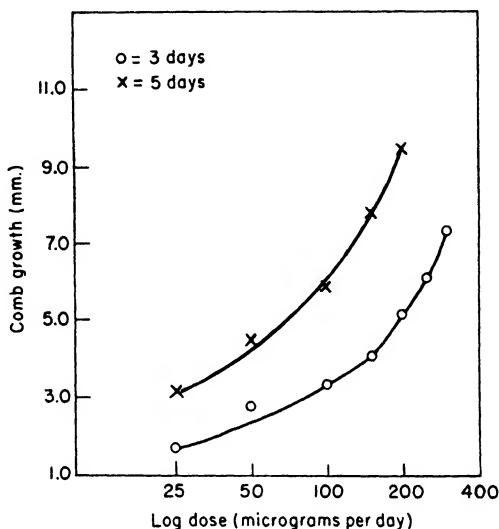
FIG. 3. Capon's comb response to androsterone injected daily for 3 and 5 days respectively (Emmens, 1939).
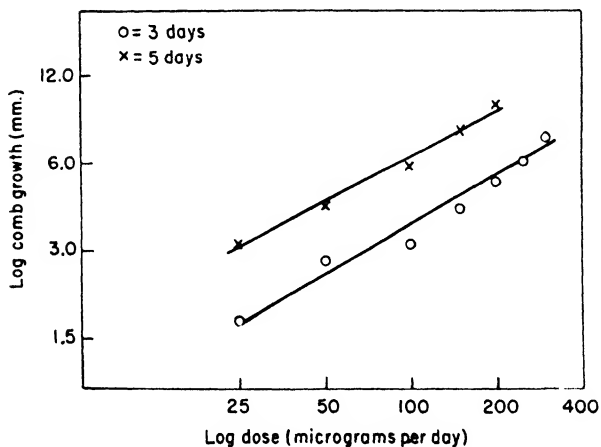
FIG. 4. Capon's comb response to androsterone injected daily for 3 and 5 days respectively (Emmens, 1939).

B. INUNCTION

i. *Method of Emmens* (*1939*). White or Brown Leghorn capons may be used. The hormone dissolved in oil is applied daily for 3 days to the
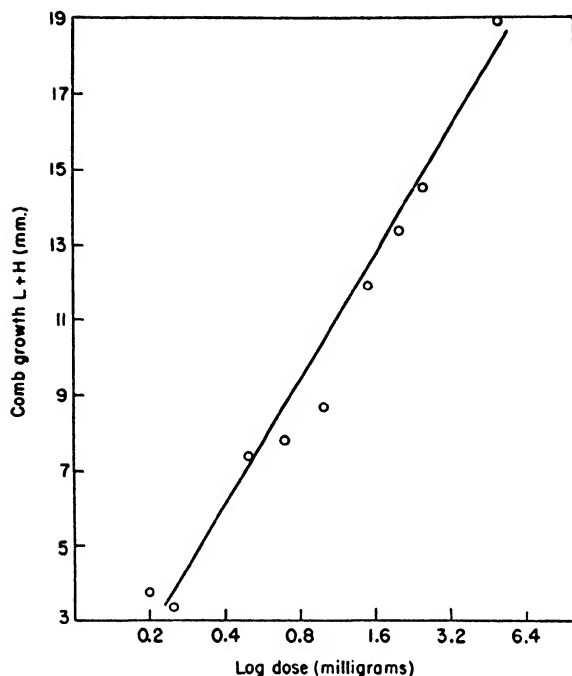
FIG. 5. Capon's comb response to androsterone injected daily for 5 days (McCullagh and Cuyler, 1939).

TABLE IV

Estimated Errors at Different Comb Growth Levels and the Standard Error of the Estimate of the Daily Dose of a Test Preparation Expressed as a Percentage of the Estimate, Assuming the Dose Response Lines to Be Accurately Known. (Emmens, 1939)

Estimated Errors

Comb growth $L + H$	Average standard errors of response, approximate. (Pooled data from 3-day and 5-day tests)			Standard error of estimate of daily Dose: %			
	σ	σ_m 5 birds	σ_m 10 birds	3-day		5-day	
				5 birds	10 birds	5 birds	10 birds
2 mm.	0.75	0.35	0.25	41.0	30		
4 mm.	1.10	0.50	0.35	17.5	12	31	21
6 mm.	1.40	0.70	0.45	14.5	10	19	12.5
8 mm.	1.75	0.85	0.60	12.5	8.5	15.5	10.5
10 mm.	2.10	1.00	0.70			13.5	9.0

capon's comb; 0.1 ml. of oil solution is used each day. Before treatment is started the combs are measured with a millimeter rule and again 24 hr. after the last inunction (see p. 293). The response length (L) plus height (H) is determined by the difference between the two measurements.

TABLE V

The Response of the Capon's Comb to Injected Androsterone by Method of Gallagher and Koch (Data of McCullagh and Cuyler, 1939)[a]

Total dose µg.	Number of birds	Mean comb growth mm. ± S.E.
50	15	1.20 ± 0.37
100	15	1.53 ± 0.43
150	14	2.44 ± 0.46
200	15	3.75 ± 0.48
250	14	3.33 ± 0.48
300	16	5.08 ± 0.42
500	16	6.50 ± 0.59
500	14	7.67 ± 0.50
500	15	7.98 ± 0.74
700	16	7.80 ± 0.77
1000	16	8.66 ± 0.96
1500	16	11.91 ± 0.77
2000	16	13.41 ± 0.95
2500	15	14.51 ± 1.14
5000	14	18.83 ± 1.02

[a] Corrected for 57-mm. comb size.

TABLE VI

The Response of the Capon's Comb to Androsterone Administered by Inunction (Data of Emmens, 1939)

Three daily inunctions

Total dose androsterone µg.	Number of capons	Comb response $L + H$ mm. ± S.E.
1.2	10	2.55 ± 0.60
2.4	10	5.30 ± 0.55
3.6	10	5.80 ± 0.78
4.8	10	7.05 ± 0.32

TABLE VII

The Assay of Androsterone by the Capon Comb Inunction Method of Emmens (1939)

Based on the data of Emmens (1939)

$N = 5$

High dose of standard = High dose of unknown
Low dose of standard = Low dose of unknown
Standard = Unknown

Dosage level µg.	b	λ	t	Potency ratio % ± S.E.
0.4; 0.8	9.33	0.108	1.215	130 ± 16
0.4; 1.2	6.88	0.360	0.684	92 ± 34
0.4; 1.6	7.50	0.186	0.875	97 ± 19
0.8; 1.6	5.83	0.079	2.610	119 ± 22

The data are those of Emmens which have been calculated by the simplified design of Bliss (1944). Table VI and Fig. 6 present the response of the capon's comb to a total dose of from 1.2 to 4.8 μg. of androsterone. Table VII presents the results of 4 theoretical assays using a total of 20 animals, 10 on the standard and 10 on the unknown.

Since the unknown and standard are identical the actual potency ratio is 100%. In the 4 tests the determined potency ratio varied from 92 ± 34 to 130 ± 10. In only one instance were the slopes of the unknown and standard significantly different. The index of precision λ varied from 0.108 to 0.360 with a mean of 0.184 ± 0.063.
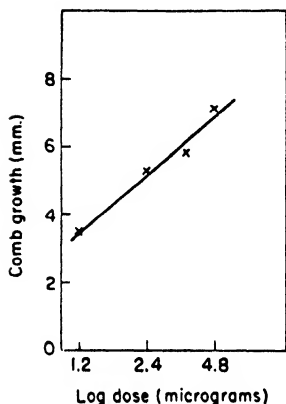
Fig. 6. Capon's comb response to androsterone administered by inunction daily for 3 days (Emmens, 1939).

ii. *Method of McCullagh and Cuyler (1939)*. This method is essentially the same as described under Gallagher and Koch (1935) except for the application of the androgen solution directly to the capon's comb. McCullagh and Cuyler (1939) have been able to demonstrate a log dose-response curve which is a straight line from a total dose of 2 μg. to 80 μg. of androsterone.

The androgen is dissolved in sesame oil so that the total dose is contained in 1 ml. of solution. Each day for 5 consecutive days, 0.2 ml. of the androgen solution is applied evenly over the whole comb.

Table VIII presents the influence of androsterone by inunction on

TABLE VIII

The Response of the Capon's Comb to Androsterone Administered by Inunction (Data of McCullagh and Cuyler, 1939)

Total dose of androsterone μg.	Number of capons	Mean comb growth mm. ± S.E.
2.0	15	1.0 ± 0.37
4.0	15	2.0 ± 0.43
7.0	15	2.53 ± 0.55
10.0	15	4.27 ± 0.52
20.0	15	7.93 ± 0.64
50.0	15	12.87 ± 0.67
100.0	15	14.33 ± 0.80

the capon's comb. The total dose ranged from 2 to 100 μg. Between the limits of 7 and 50 μg. an excellent agreement was found for a linear
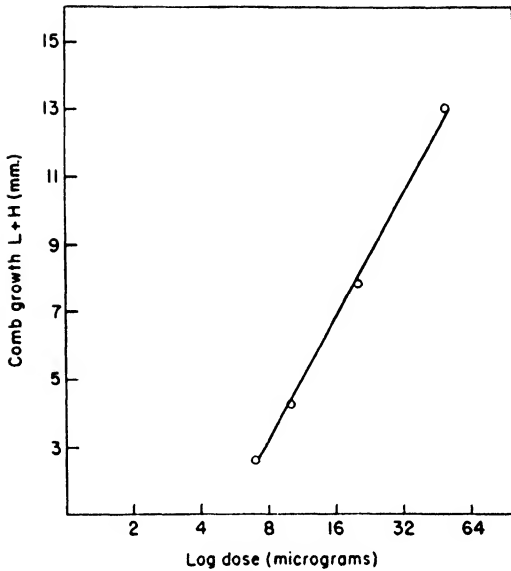
Fig. 7. Capon's comb response to androsterone administered by inunction daily for 5 days (McCullagh and Cuyler, 1939).

relationship between the logarithm of the dose and the response (Fig. 7). The slope over this range was found to be 30.3 and $\lambda = 0.0763$.

2. Chick Comb Growth

A. GENERAL REMARKS

The early studies of Ruzicka (1935), Burrows *et al.* (1936), Dorfman and Greulich (1937), and Frank *et al.* (1937) indicated the advisability of using the chick's comb as the test object for androgen assays. Ruzicka (1935) painted the chick's comb with a 0.5% solution of androsterone in oil each day for a period of several weeks, and obtained large increases in comb area. He did not, however, study this reaction quantitatively. Frank and Klempner (1937) applied the androgens in oil solutions directly to the base of the comb of White Leghorn chicks. Applications were begun on the sixth day after hatching and were repeated on 10 successive days. The animals were sacrificed and the comb weights were determined on the day following the last application. These workers were able to evoke a definite response with as little as 20 μg. of androsterone. Burrows and his co-workers (1936) injected both androsterone and testosterone either into the base of the chick's comb or into the breast muscles and found that both of these androgens stimulated comb growth. In all the studies mentioned the response is the weight of the comb, which

perhaps represents an advantage over the less exact methods of measurement of the size of the capon's comb. However, the capon comb method has the advantage that each animal serves as its own control, since comb measurements are made before and after hormone administration.

B. RELATIVE REACTIVITY OF THE COMB OF VARIOUS BREEDS OF CHICKS TO ANDROGENS (DORFMAN, 1948b)

It has been known for some time that various breeds of fowl differ as to their practicability for use in androgen assays when the comb is used as the test organ. Recently, the reactivity of 3 breeds of chicks to testosterone propionate has been studied. The hormone was administered by direct application to the comb. This procedure should rule out variations in the metabolism or inactivation of the hormone in the body. In these experiments we are dealing only with the direct stimulation and the local inactivation of the hormone at the site of the comb.

The 3 breeds of chicks studied were the White Leghorn, the Rhode Island Red, and the Barred Rock. The animals were kept in a thermostatically controlled brooder and were fed chick starting mash and water exclusively. The chicks were 2 to 3 days of age at the beginning of the experiment. The total dose of testosterone propionate was contained in 0.35 ml. of corn oil and administered once daily for 7 days. Five-hundredths of a milliliter of the hormone solution was dropped on the comb from a 1-ml. tuberculin syringe fitted with a number 24 hypodermic needle. Twenty-four hours after the last hormone application the animals were killed, and body weight and comb weights were determined. The animals were autopsied at 9 to 10 days of age. The comb responses are expressed as the ratio of the comb weight in milligrams to the body weight in grams. Body weights did not vary significantly.

The dosage range of testosterone propionate investigated varied from 2 μg. to 20,480 μg. for the White Leghorn male and female chicks, and 40 μg. to 20,480 μg. for the Rhode Island Red and Barred Rock breeds (Table IX).

No significant difference was found in the comb ratios between male and female untreated chicks for any of the 3 breeds studied, but a significant difference in comb ratios was found between animals of different breeds. White Leghorn chicks showed the largest combs, the Barred Rocks the smallest, and the combs of the Rhode Island Reds were intermediate. This order of comb size of control chicks was true for both the males and females.

On the basis of the minimal quantity of testosterone propionate needed to produce a 20% increment in comb ratio, the male White Leghorns were 15 times as sensitive as the Rhode Island Reds and 20 times

as sensitive as the Barred Rocks. Similarly, the female White Leghorn combs were 10 times as sensitive as those of the Rhode Island Reds, and 20 times those of the Barred Rocks.

TABLE IX

The Relative Reactivity of the Combs of White Leghorn, Rhode Island Red, and Barred Rock Chicks to Testosterone Propionate When Administered by Direct Application to the Comb

Breed of chick	Amount administered µg.	Number of chicks M	F	Ratio $= \dfrac{\text{Comb in mg.}}{\text{B.W. in g.}} \pm$ S.E. M	F
White Leghorn	0	62	56	0.38 ± 0.01	0.36 ± 0.01
	2	13	6	0.46 ± 0.04	0.43 ± 0.06
	5	28	34	0.55 ± 0.02	0.47 ± 0.02
	10	32	31	0.63 ± 0.03	0.52 ± 0.02
	20	18	45	0.65 ± 0.04	0.67 ± 0.02
	40	36	51	0.77 ± 0.05	0.69 ± 0.02
	80	46	38	1.03 ± 0.05	0.91 ± 0.05
	160	29	36	1 53 ± 0.10	1.54 ± 0.09
	2560	25	13	1.74 ± 0.07	1.74 ± 0.15
	5120	12	12	1.88 ± 0.15	1.98 ± 0.15
	20,480	8	13	1.89 ± 0.22	1.82 ± 0.16
Rhode Island Red	0	47	29	0.25 ± 0.01	0.23 ± 0.01
	40	14	11	0.33 ± 0.01	0.33 ± 0.02
	80	13	11	0.36 ± 0.03	0.40 ± 0.04
	160	9	14	0.48 ± 0.05	0.49 ± 0.05
	640	9	16	0.85 ± 0.10	0.82 ± 0.10
	1280	15	10	0.91 ± 0.07	0.87 ± 0.09
	2560	32	21	0.92 ± 0.05	1.03 ± 0.07
	5120	11	12	1.14 ± 0.07	1.03 ± 0.08
	20,480	..	19	....	1.10 ± 0.08
Barred Rock	0	36	37	0.21 ± 0.01	0.20 ± 0.01
	40	13	10	0.25 ± 0.02	0.22 ± 0.02
	80	12	13	0.23 ± 0.02	0.29 ± 0.02
	160	9	14	0.36 ± 0.04	0.32 ± 0.02
	640	12	17	0.54 ± 0.03	0.55 ± 0.03
	1280	11	15	0.61 ± 0.02	0.63 ± 0.04
	2560	24	31	0.85 ± 0.01	0.82 ± 0.04
	5120	9	15	0.97 ± 0.09	1.07 ± 0.08
	20,480	12	10	1.01 ± 0.11	1.02 ± 0.07

The comparative sensitives of the combs of the 3 breeds were evaluated by selecting portions of the log dose-response curves where the slopes of all 3 breeds were not significantly different, and by using the displacement of the curves as another measure of the relative sensitivity of the various breeds (Table X and Figs. 8 and 9). Under these conditions and expressing the sensitivity of the male White Leghorn chick comb as 100%, the sensitivity of the Rhode Island Red was found to be 10% and that of

the Barred Rock 1.8%.   In the female chicks the relative comb sensi-
tivities were similarly White Leghorn, 100%; Rhode Island Red, 8.9%;
and Barred Rock 1.8% (Table XI).
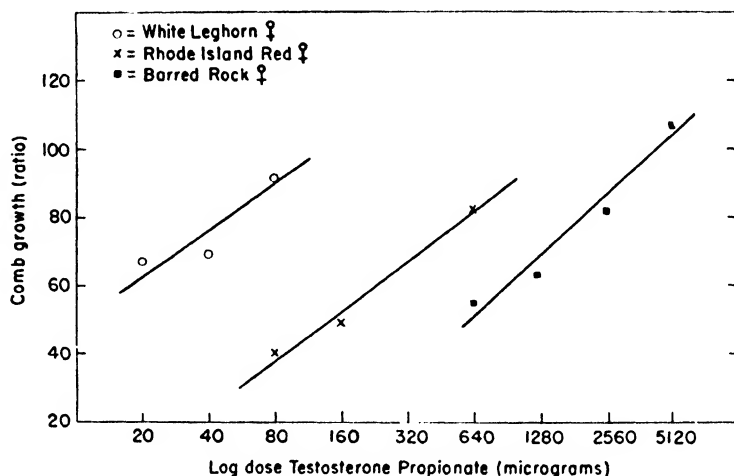
FIG. 8.   Comb responses of three breeds of male chicks to testosterone propionate
(Dorfman, 1948b).
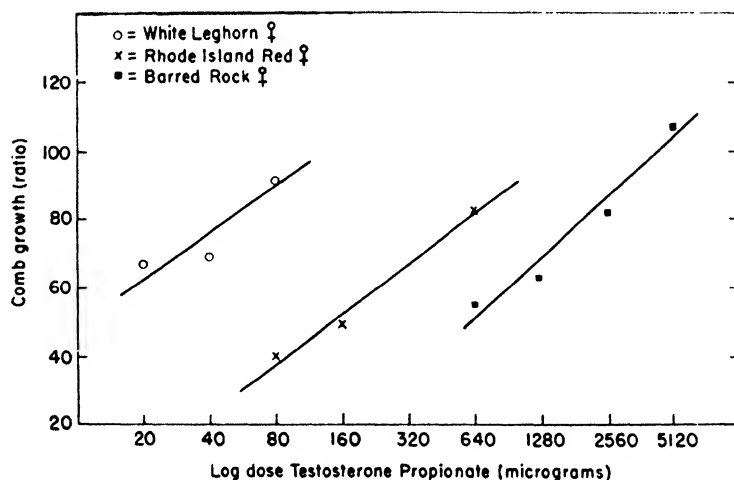
FIG. 9.   Comb responses of three breeds of female chicks to testosterone propionate
(Dorfman, 1948b).

A third criterion of sensitivity of the combs to androgen was the
maximum slope attainable using a log dose-response relationship for at
least 3 points.   No significant difference in maximum slope was found

TABLE X

The Maximum Slopes of Comb Response Attainable by Rhode Island Red and Barred Rock Chicks[a]

(Logarithm dose-response)

Breed of chicks	Sex	Dosage levels of testosterone propionate μg.	Total number of animals	Slope $b \pm$ S.E.
White Leghorn[a]	M	20; 40; 80	100	0.677 ± 0.107
	F	10; 20; 40; 80	165	0.357 ± 0.023
Rhode Island Red	M	80; 160; 640	31	0.545 ± 0.026
	F	80; 160; 640	41	0.485 ± 0.110
Barred Rock	M	640; 1280; 2560	47	0.537 ± 0.101
	F	640; 1280; 2560; 5120	78	0.484 ± 0.110

[a] The White Leghorn comb response curve was taken where the slope was not significantly different from that of the other breeds.

TABLE XI

Relative Sensitivity of Various Breeds to Testosterone Propionate as Measured by Amount of Hormone Necessary to Produce Similar Slopes

Breed of animals	Males		Females	
	Sensitivity in % of White Leghorn	Error range $P = 0.95$	Sensitivity in % of White Leghorn	Error range $P = 0.95$
White Leghorn	100	0	100	. . . .
Rhode Island Red	10.0	+32; −25	8.9	+48; −33
Barred Rock	1.8	+23; −19	2.0	+33; −25
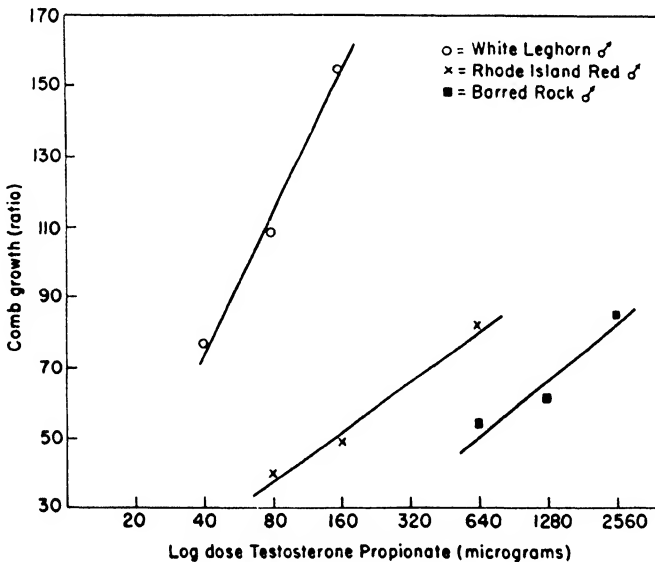
FIG. 10.   Comb responses of three breeds of male chicks to testosterone propionate (Dorfman, 1948b).

for the Rhode Island Red and Barred Rock chick combs, but the White Leghorns showed a significantly greater slope (Table XII and Fig. 10).

TABLE XII

Comparison of Maximum Slopes Attained by Regression of White Leghorn and Barred Rock Combs on Testosterone Propionate[a]

Sex	Maximum White Leghorn slope[b] ± S.E.	Maximum Barred Rock slope ± S.E.	Total number of chicks	t	P
M	1.215 ± 0.147	0.537 ± 0.101	158	3.027	0.01
F	1.361 ± 0.100	0.484 ± 0.110	223	8.240	0.01

[a] Rhode Island Red maximum slopes not significantly different from those of Barred Rock.
[b] Derived from 40-, 80-, and 160-μg. doses of testosterone propionate.

Finally, an evaluation of the maximum percentage increase in comb size by androgen stimulation revealed that no significant difference could be demonstrated between the 3 breeds for either male or female chicks (Table XIII).

TABLE XIII

Comparative Maximum Comb Ratios Attained by Three Breeds of Chicks after Application of Testosterone Propionate

Breed of chick	Males		Females		Mean increase male and female %
	Control Ratio	Maximum increase %	Control ratio	Maximum increase %	
White Leghorn	0.38	395	0.36	415	405
Rhode Island Red	0.25	357	0.23	364	361
Barred Rock	0.21	344	0.20	426	385

C. METHOD OF FRANK *et al.* (1942)

The method was devised by Frank *et al.* (1942) and studied by Dorfman (1948a). Using it, Klempner *et al.* (1942) have shown that in 24 determinations of androsterone in the dosage range of 20 to 40 μg., the mean error was 13%, and in 39 determinations over the range of 10 to 50 μg., the mean error was 24.6%. In another study, (Dorfman, 1948a) the results of Klempner *et al.* (1942) were confirmed. In the latter study, in the range of 20 to 40 μg., a mean error of 12% was found, and in the range of 10 to 40 μg., a mean error of 24% was found.

White Leghorn chicks should be used at 2 to 3 days of age. Mixed pullets and cockerels are used. The animals should be kept in a brooder with a thermostatic control. It is well to keep the temperature between 88° and 96°F.

The total dose of material to be administered to each chick is dissolved in 0.35 ml. of sesame oil; 0.05 ml. of oil is administered daily for

7 days, starting when the animals are 2 to 3 days of age. The material is dropped on the comb by means of a 1-ml. tuberculin syringe fitted with a fine hypodermic needle. An attempt is made to apply the oil solution slowly so that spreading to the head feathers is minimized. Twenty-four hours after the last application of androgen solution, the chicks are autopsied (8–9 days of age).

Body weights are determined at the time of the first application of the androgens, and again at autopsy along with weight of comb and sex of the animal. The combs are removed by two longitudinal incisions along the base of the comb at its juncture with the scalp. The incisions are extended vertically down to the skull. The comb is freed from the skull and the base is touched lightly on a towel to remove blood from the cut surface. The comb is weighed quickly on a suitable torsion balance to avoid drying.

Calculations:

Activity equivalent to 100 μg. of androsterone per chick

$$= \frac{1.061(\Sigma W) - 0.0043(\Sigma W^2) - 0.397(\Sigma B_i) - 0.267(\Sigma B_t) + 14.75 N_m + 18.54 N_f}{100(N_m + N_f)}$$

where ΣW = the sum of the comb weights, expressed in mg.

ΣW^2 = the sum of the squared comb weights

ΣB_i = the sum of the initial body weights, expressed in g.

ΣB_t = the sum of the terminal body weights, expressed in g.

N_m = the number of male chicks used in the assay

N_f = the number of female chicks used in the assay.

D. METHOD OF DORFMAN (1948a) (INUNCTION)

i. *Testosterone Propionate.* The assay of testosterone propionate can be carried out by a chick comb method using the details of age of chicks, volume of oil, time of hormone application to comb, and determination of the comb weight as described on p. 306. The experimental design and calculations are different.

The design of Bliss (1944) is employed, using 2 concentrations of the standard and 2 concentrations of the unknown. The total concentration of testosterone propionate used for each animal should be in the range of 20 to 160 μg. Within the range of 20 to 160 μg., using 32 animals on the standard and 32 animals on the unknown, errors in the determination of the potency ratio are lower than 38% at $P = 0.95$.

The response of the male and female White Leghorn chick's comb to testosterone propionate is presented in Table XIV. In Table XV the data have been considered by the method of Bliss (1944). A linear relationship was found when the logarithm of the dose was plotted

against the logarithm of the response, and the data are calculated on this basis.   In each instance, 2 dose levels of both the unknown and standard were considered.   The number of animals in each group in 3 cases was

TABLE XIV

The Response[a] of the Chick Comb to Testosterone Propionate

5 μg.		10 μg.		20 μg.		40 μg.		80 μg.		160 μg.	
S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂
0.45	0.48	0.38	0.98	0.44	0.51	1.10	0.96	1.33	1.13	2.46	2.16
0.51	0.52	0.56	0.73	0.58	0.69	0.67	1.10	1.10	1.31	1.62	1.38
0.63	0.68	0.53	0.84	1.13	0.50	0.81	0.80	0.97	1.30	1.60	2.71
0.67	0.50	0.75	0.71	1.02	0.56	0.81	0.89	1.17	0.77	1.30	3.21
0.75	0.62	0.66	0.67	0.58	0.41	1.37	1.52	1.64	1.34	1.15	1.13
0.48	0.67	0.66	0.53	0.55	0.59	0.90	0.61	1.55	1.48	1.25	2.00
0.69	0.59	0.42	0.68	0.82	0.69	0.71	0.45	1.11	1.46	1.72	1.17
0.50	0.47	0.77	0.56	0.76	0.44	0.63	1.08	1.48	1.11	1.23	1.91
S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀
0.43	0.37	0.43	0.61	0.63	0.83	0.36	0.64	0.76	1.15	0.85	1.53
0.32	0.32	0.38	0.41	0.68	0.84	1.68	0.69	0.92	1.42	2.03	1.09
0.53	0.58	0.54	0.55	0.57	0.69	0.46	0.93	0.96	0.88	2.65	1.63
0.37	0.67	0.56	0.55	0.55	0.73	0.69	0.89	0.84	0.51	0.89	1.60
0.45	0.62	0.80	0.48	0.92	0.82	0.78	0.73	0.82	1.16	2.12	2.05
0.39	0.37	0.43	0.36	0.66	0.51	0.84	1.69	1.34	1.20	2.76	2.66
0.67	0.49	0.51	0.49	0.58	0.43	0.82	0.97	0.96	1.13	1.59	1.98
0.47	0.38	0.49	0.42	0.65	0.53	0.86	0.76	0.76	1.21	1.14	1.90

[a] Response expressed as ratio of comb weight in milligrams to body weight in grams.

TABLE XV

Assay of Testosterone Propionate by Direct Comb Application

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown

(log dose = log response)

N (half ♂ half ♀)	Dosage levels μg.	b	λ	t	Potency ratio % ± S.E.	Error range P = 0.95 %
16	40; 160	0.5025	0.277	0.318	128 ± 21	−27; +38
12	40; 160	0.5000	0.322	1.835	129 ± 24	−31; +46
16	20; 80	0.4115	0.229	0.805	87 ± 14	−27; +38
12	20; 80	0.3729	0.329	0.785	95 ± 20	−35; +54
16	80; 160	0.5908	0.205	1.445	111 ± 13	−21; +26
16	20; 40	0.3848	0.369	0.694	102 ± 23	−38; +56

16 (8 males and 8 females), and 12 (6 males and 6 females) in the other comparisons.

At the level of 40 to 160 μg. for groups of 16 (total animals, 64) an error range of −28 to +38% was found as compared to an error range

of -31 to $+46\%$ for groups of 12 (total animals, 48). In a second instance where groups of 16 and 12 were compared at the 20 and 80 μg. levels, an error range of -27 to $+38\%$ was found for the groups of 16, while the groups of 12 showed an error range of -35 to $+54\%$ ($P = 0.95$).

The highest error range of the potency ratio was found at the levels of 20 and 40 μg., although 16 animals were used at each dose level. This error range was from -38 to $+56\%$.

TABLE XVI

Response of the Male Chick's Comb to Testosterone Administered by Application[a]
(Dorfman, 1949)

Total amount administered μg.	Number of chicks	Comb ratio $\pm$ S.E.
0	62	38 $\pm$ 1
50	33	84 $\pm$ 4
100	32	112 $\pm$ 5
200	34	136 $\pm$ 7

[a] Seven days.

No significant difference was found in the slopes of the unknown and standard. The t values ranged from 0.318 to 1.835.

ii. *Testosterone.* Testosterone was assayed by the same procedure used for testosterone propionate. A linear relationship was found

TABLE XVII

Assay of Testosterone by Direct Comb Application (Dorfman, 1949)

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

$$\text{Standard} = \text{Unknown}$$
$$N = 15$$

Dosage levels μg.	b	λ	t	Potency ratio $\pm$ S.E.
50; 100	105	0.238	0.145	92 $\pm$ 13
100; 200	87	0.415	1.510	124 $\pm$ 30
100; 200	137	0.251	1.840	94 $\pm$ 11
100; 200	102	0.360	1.160	102 $\pm$ 16
100; 200	86	0.408	0.684	129 $\pm$ 32

between the logarithm of the dose and the response. The simplified design was employed (Bliss, 1944).

Table XVI and Fig. 11 illustrate the response of the male chick's comb to the direct application of testosterone. The assay is illustrated

in Table XVII. The actual potency ratio was 1. The potency ratios varied from 0.92 to 1.29 in 5 different runs. No significant differences in the slopes of the unknown and standard were found.
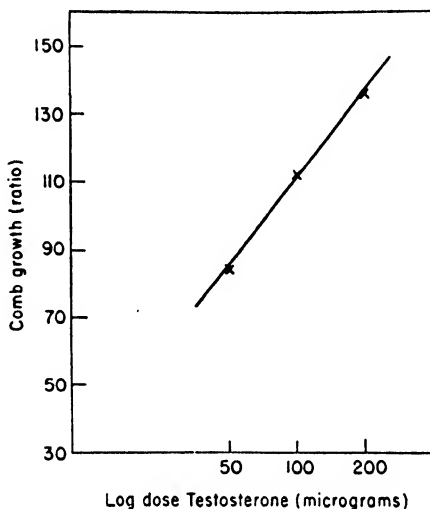
FIG. 11. Chick's comb response to testosterone administered by direct application (Dorfman, 1949).
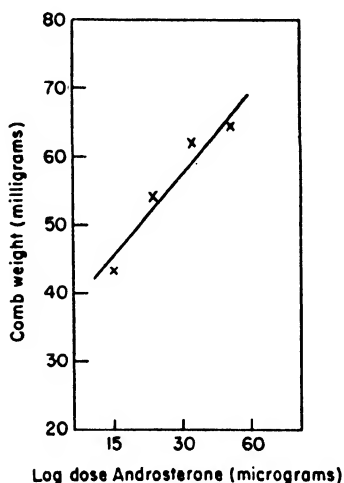
FIG. 12. Chick's comb response to androsterone administered by direct application (Valle *et al.*, 1947).

iii. *Androsterone Valle et al. (1947).* Valle *et al.* (1947) have studied the chick comb method by direct application of androsterone. Two-day-old White Leghorn male chicks are placed in brooders at a tempera-

ture of 32° to 37°C. They are fed *ad libitum* bread and milk plus a dry food consisting of corn meal, 50%; rice bran, 20%; wheat bran, 15%; meat meal, 10%; and bone meal, 5%. Beginning with the third day of life and continuing for seven consecutive days, 0.05 ml. of oil solution of the hormone is applied to the comb. Twenty-four hours after the last hormone application the animals are killed and the combs removed by the method of Frank *et al.* (1942), described on p. 306. Animals which gained less than 2 g. during the experimental period were discarded.
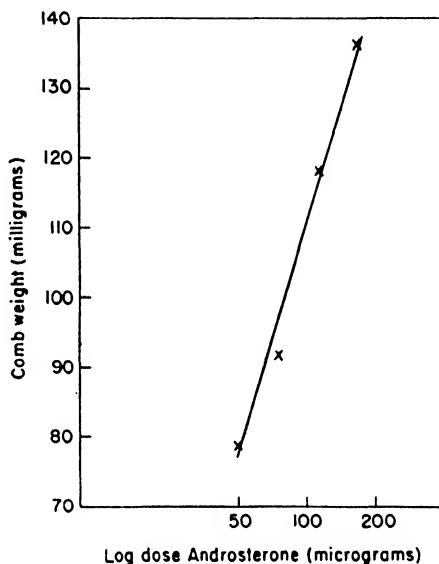
FIG. 13. Chick's comb response to androsterone administered by direct application (Valle *et al.*, 1947).

Table XVIII and Figs. 12 and 13 illustrate the results using androsterone by direct application. In experiments *A* through *D* low concentrations (15 to 50 μg.) of androsterone were applied. Under these conditions a mean slope of 44.1 ± 3.68 was found and a mean value of λ = 0.424 ± 0.033 was calculated. Between 50 and 168 μg. of androsterone, a steeper slope of 115.114 was found and a correspondingly more favorable value of 0.244 for λ.

E. METHOD OF DORFMAN (1948b) (INJECTION)

Testosterone propionate dissolved in corn oil is injected subcutaneously once daily for 5 consecutive days, starting 4 days after hatching. Male White Leghorn chicks are used. The daily dose is contained in 0.1 ml. of oil. Twenty-four hours after the last injection the chicks are

TABLE XVIII

The Response of the Male Chick's Comb to Androsterone by Application

Experiment No.	Dosage μg.	Number of chicks	Mean comb weight mg.	Variance	Slope	Sigma of slope
A	15.0	20	46.7	267.95		
	22.4	20	53.1	272.77	42.264	11.54
	33.5	19	62.0	699.82		
	50.0	18	68.3	408.86		
B	15.0	18	41.9	247.62		
	22.4	14	49.0	218.30	41.685	10.56
	33.5	16	56.3	261.76		
	50.0	16	63.7	652.37		
C	15.0	14	55.2	305.41		
	22.4	16	60.9	389.34	41.516	11.42
	33.5	16	67.3	360.17		
	50.0	12	77.2	599.61		
D	15.0	17	42.5	204.34		
	22.4	20	48.9	253.78	55.975	10.04
	33.5	14	64.0	384.08		
	50.0	18	70.0	408.50		
E	50.0	17	78.8	606.74		
	75.0	16	91.8	797.63	115.114	16.14
	112.0	18	118.1	949.14		
	168.0	16	136.1	794.86		

killed with chloroform, and the comb and body weights determined. The combs are removed as described on p. 306. The response is expressed as 100 times the ratio of the comb weight in milligrams to the body weight in grams. A linear relationship is found when the logarithm of dose is plotted against the response.

The results of three tests using the simplified design are shown in Table XIX. The actual potency ratio was 1. Potency ratios of 0.92

TABLE XIX

Assay of Testosterone Propionate by Injection (Dorfman, 1949)

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown

$N = 15$

Dosage level mg.	b	λ	t	Potency ratio % ± S.E.
0.5; 1.0	47.5	0.374	1.278	92 ± 20
1.0; 2.0	83.9	0.276	0.106	120 ± 20
0.5; 2.0	66.2	0.275	1.865	102 ± 17

± 0.20, 1.20 ± 0.20, and 1.02 ± 0.17 were found.   The slopes of the standards and unknowns did not differ significantly.   The measure of precision, λ, was 0.374, 0.276, and 0.275.

F. METHOD OF DORFMAN (1949) (ORAL ADMINISTRATION)

The hormone is mixed thoroughly with a standard chick starting mash in a mechanical mixer.   White Leghorn cockerels are fed this
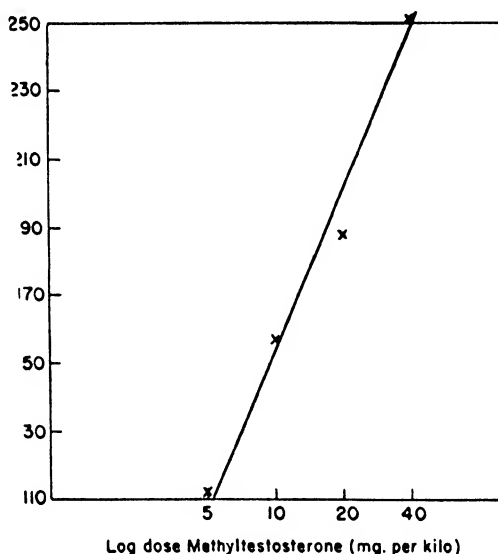
FIG. 14.   Chick's comb to methyltestosterone administered orally (Dorfman, 1949).

mixture for 10 days starting at 2 to 3 days of age.   The chicks are killed with chloroform, at which time comb and body weights are determined. The removal of the combs is described on p. 306.   The response is repre-

TABLE XX

The Response of the Male Chick's Comb to Methyltestosterone Administered Orally (Dorfman, 1949)[a]

Conc. of androgen in food mg./kg.	Number of chicks	Comb ratio ±S.E.
0	32	42 ± 2
5	23	112 ± 8
10	23	157 ± 11
20	20	188 ± 14
40	19	251 ± 17

[a] Ten days on diet.

sented as 100 times the ratio of the comb weight in milligrams to the body weight in grams. A linear relationship exists between the logarithm of the dose and the response.

TABLE XXI

The Assay of Methyltestosterone Administered Orally to Male Chicks (Dorfman, 1949)

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown
(Log dose = response)
N = 10

Dosage levels mg./kg.	b	λ	t	Potency ratio % ± S.E.
5; 10	151	0.315	1.060	129 ± 30
5; 20	122	0.404	0.266	121 ± 35
30; 40	207	0.326	0.650	83 ± 20

The response of the chick's comb to orally administered methyltestosterone is shown in Table XX and Fig. 14. Table XXI shows the results of the assay using the simplified design (Bliss, 1944).

3. Sparrow's Bill (Pfeiffer et al., 1944)

Androgens cause blackening of the bill of the English sparrow. Thus, the male in the winter months has an ivory-colored bill, and as the testis function increases in the spring the bill blackens. This blackening is due to the increased concentration of androgen resulting from the increased testicular function, and has been suggested for the assay of androgens but as yet has not been adapted to quantitative assay. Some of the results of Pfeiffer et al. (1944) are presented to illustrate the potentialities of the method.

English sparrows (*Passer domesticus*) were trapped as immature birds in summer. The males were castrated and females were used as intact animals in the fall of the year. The animals were housed in wire cages measuring 3x3x4 ft. A dry mixture of finely cracked grain and poultry growing mash was fed along with an ample supply of fresh water. In the experiments presented in Tables XXII, XXIII, and XXIV, some animals received the hormone by direct application to the breast; others received the hormone subcutaneously; while still others received the hormone by direct application to the bill. The hormone was given intramuscularly in 0.05 ml. of sesame oil per day, or was applied to the skin in the breast region in absolute alcohol. The local bill reaction was produced by placing one drop of an absolute alcohol solution on one

TABLE XXII

The Response of the Sparrow's Bill to Testosterone Propionate Administered Intramuscularly (Pfeiffer et al., 1944)

Daily dose µg.	Number of birds and sex	Number of days	Animals responding %
2.5	4	10	0
5.0	4	10	0
10.0	4	10	100
2.5	3	10	0
5.0	3	10	67
10.0	3	10	100

TABLE XXIII

The Response of the Sparrow's Bill to Testosterone Propionate Applied to the Skin of the Breast (Pfeiffer et al., 1944)

Daily dose µg.	Number of birds and sex	Number of days	Animals responding %
2.0	3	25	33
4.0	5	25	60
8.0	4	15	75

TABLE XXIV

The Response of the Sparrow's Bill to Testosterone and Androsterone Applied Locally (Pfeiffer et al., 1944)

Androgen	Daily dose µg.	Number of birds and sex	Number of days	Animals responding %
Testosterone	0.5	5	10	60
	1.0	2	10	100
	2.0	2	10	100
	0.063	6	16	67
	0.125	3	10	100
	0.250	4	10	100
Androsterone	0.25	4	10	100
	0.50	4	10	100
	1.00	4	10	100
	3.30	4	10	100
	6.60	4	10	100
	13.30	3	10	100
	0.063	5	16	100
	0.125	5	10	60
	0.250	10	10	100

side of the bill from a number 22 hypodermic needle fitted to a 1-ml. tuberculin syringe.

Table XXII presents the response of the Sparrow's bill to testosterone propionate administered intramuscularly. A total of 100 μg. administered over 10 days produced 100% responses in both castrated males and normal females. A total of 50 μg. in the same period of time produced blackening in 2 of 3 castrated males. The application of the hormone to the skin in the breast area was not particularly effective (Table XXIII). However, direct application of androgens, either androsterone or testosterone, was the most sensitive test yet described. A total of 1 μg. of testosterone administered in 16 divided doses produced positive responses in 4 of 6 castrated males, and a similar dose of androsterone produced 100% of positive responses in 5 castrated males (Table XXIV).   .

4. Additional Bird Methods

In addition to the methods already described, reports on the use of the capon's comb by injection have been made by Ruzicka *et al.* (1934), Tschopp (1935), Butenandt and Tscherning (1934), and Dingemanse (1931). Various methods using the direct comb application of androgens have been described by Dessau (1935, 1937), Voss (1937), Fussganger (1934), Oesting and Webster (1938), Mussio Fournier *et al.* (1940). Courier and Jost (1939) have published studies on the use of the chick comb method.

IV. MAMMALIAN ASSAYS

1. Method of Mathison and Hays (1945)

The standard and unknown should be run simultaneously at each of two concentrations so that the ratio of the high dose to the low dose of both unknown and standard is equal to 4. For this purpose the high dose will be taken as the material supplied, whereas the low dose will be prepared by diluting the material supplied in one part plus 3 parts of corn oil.

Rats are castrated between the ages of 26 to 29 days and weighing 40 to 75 g. The animals are used between 2 to 12 weeks after castration at which time the animals are divided into 4 groups, each group containing 12 castrated animals.

Each castrated rat receives 0.2 ml. of a solution containing either the unknown or standard. The solutions are injected subcutaneously using a 1-ml. tuberculin syringe fitted with a 24-gauge needle. Seventy-two hours after the administration of the test material the rats are

killed and the body weight is determined. The ventral surface is opened to expose the male accessory organs and bladder. Two lateral incisions are made to permit easy access to the seminal vesicles. With a pair of forceps and curved iridectomy scissors, each of the vas deferens is cut and the seminal vesicles and prostate removed by incising at a point near the base of the bladder. The tissue is dipped in physiological saline and placed on a cork board under a dissecting microscope. The coagulating glands are teased from the seminal vesicle and the latter incised at a point nearest the ejaculatory ducts. The seminal vesicles are again immersed in saline, dried on blotting paper for a few seconds, and weighed to the nearest 0.5 mg.

TABLE XXV

The Assay of Testosterone Propionate Using the Response of the Seminal Vesicles of the Castrated Male Rat

$$\frac{\text{High dose (unknown)}}{\text{Low dose (unknown)}} = \frac{\text{High dose (standard)}}{\text{Low dose (standard)}}$$

N	b	s	λ	t	Potency ratio $\pm$S.E. %	Theoretical potency ratio %
11	19.6	5.38	0.296	0.105	63 $\pm$ 13	80
11	20.7	5.26	0.255	1.078	80 $\pm$ 14	80
11	17.1	5.63	0.329	0.990	95 $\pm$ 22	100
11	21.4	4.68	0.218	0.679	109 $\pm$ 16	100
11	23.6	5.00	0.212	1.822	98 $\pm$ 14	120
11	18.7	4.75	0.254	0.493	133 $\pm$ 30	120

The results are listed in Table XXV. Previously, Mathison and Hays (1945) reported two assays using a solution of testosterone propionate containing 10 mg. of the hormone ester per milliliter. They found 10.72 $\pm$ 1.83 and 9.26 $\pm$ 1.74, respectively.

2. Data of Callow and Deanesly (1935)

The data of Callow and Deanesly (1935) are shown in Tables XXVI and XXVII, and Figs. 15 and 16. Table XXVI and Fig. 15 illustrate the response of the seminal vesicles and prostate to androsterone in prepuberally castrated rats treated daily for 10 days. Table XXVII and Fig. 16 present data about the seminal vesicle and prostate response of the postpuberally castrated rat to androsterone.

The postpuberally castrated animals were operated at 130 to 155 g. body weight and injected with androsterone in arachis oil for 14 consecutive days. Twenty-four hours after the last injection the animals were killed. The prepuberally castrated animals were operated at

TABLE XXVI

The Response of the Prepuberally Castrated Rat Accessory Glands to Androsterone[a]
(Callow and Deanesly, 1935)

Total dose of androsterone mg.	Number of rats	Seminal vesicles mg.	Prostate mg.
3.5	5	9	44
6.0	6	14	64
10.0	5	32	112
15.0	5	17	129
20.0	5	26	129
28.0	5	46	394

[a] Ten days' treatment.

35 to 70 g. body weight, usually between 40 to 50 g. These castrates were treated once daily with the hormone dissolved in 0.1 ml. of arachis oil for 10 consecutive days. Twenty-four hours after the last injection the animals were killed.
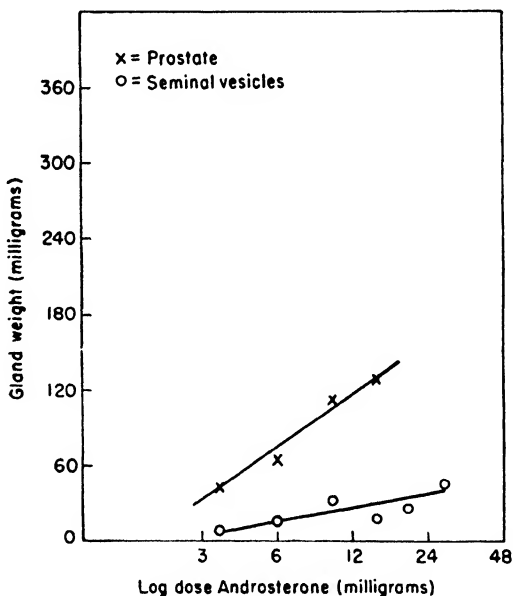
FIG. 15. Responses of prostate and seminal vesicles to androsterone administered over a 10-day period (Callow and Deanesly, 1935).

The entire reproductive tract was dissected free of fat, fixed in Bouin's fluid, and transferred to 70% alcohol. The prostates and seminal vesicles were dissected carefully, drained thoroughly, and weighed.

TABLE XXVII

The Response of the Postpuberally Castrated Rat Accessory Glands to Androsterone[a]
(Callow and Deanesly, 1935)

Total dose of androsterone mg.	Mean body weight g.	Weight of seminal vesicles mg.	Weight of prostate mg.
0	165	51	55
7.0	157	122	291
14.0	155	204	429
28.0	167	431	577

[a] Fourteen days of injection; 5 animals per group.
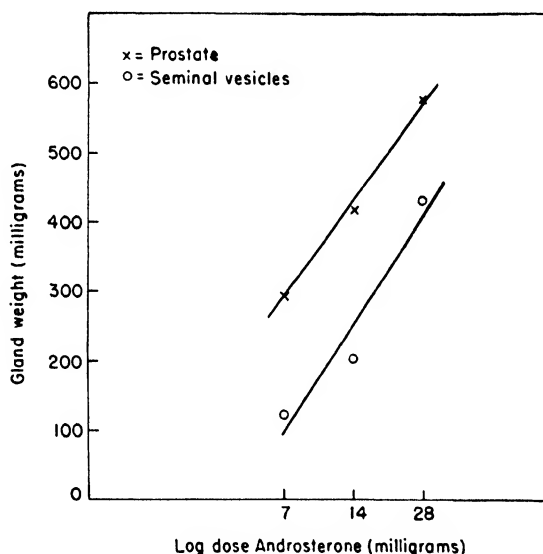
FIG. 16.   Responses of prostate and seminal vesicles to androsterone administered over a 14-day period (Callow and Deanesly, 1935).

3. Data of Miescher et al. (1936)

Male rats weighing 60 to 80 g. are used 25 to 30 days after operation. The rats receive, subcutaneously, the androgen in 0.5 ml. of sesame oil daily for 10 consecutive days.   On the eleventh day the animals are killed, and the seminal vesicle and prostate weights are determined.

Table XXVIII and Fig. 17 illustrate the responses of both the seminal vesicles and prostate to androsterone and testosterone.   No errors could be calculated from the data available.

TABLE XXVIII

The Response of the Rat Seminal Vesicles and Prostate to Androgens[a] (Data of Miescher et al., 1936)

Androgen	Total dose mg.	Number of rats	Mean weight of seminal vesicles mg.	Mean weight of prostate mg.
None	0	3	14	41
Androsterone	5	2	68	131
	20	2	142	260
Testosterone	0.5	4	40	70
	1.0	4	62	86
	2.0	4	124	176
	5.0	2	127	173

[a] Ten days' consecutive subcutaneous injection.
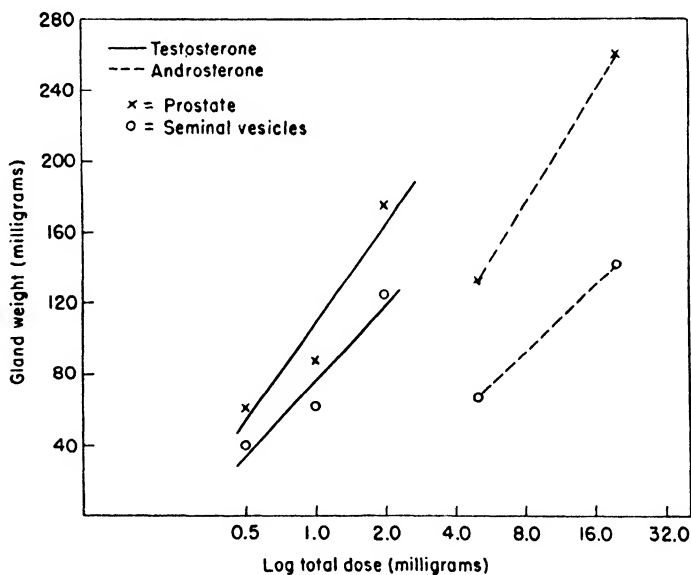
FIG. 17. Responses of prostate and seminal vesicles to androsterone and testosterone (Miescher et al., 1936).

4. Additional Mammalian Methods

A variety of investigators have reported on the use of the rodent for androgen assay. These include Tscherning (1936), Butenandt and Hanisch (1935), Morato Manaro (1940), Deanesly and Parkes (1936), Miescher et al. (1937), Korenchevsky et al. (1935), Ruzicka and Rosenberg (1936), Fischer (1938), Loewe and Voss (1931), Dirscherl et al.

(1936), Masson *et al.* (1942), Selye and Albert (1942), and Korenchevsky and Dennison (1936).

V. Summary of Methods

Tables XXIX, XXX, and XXXI summarize the characteristics of various methods for the assay of androgens. In these tables the androgen, the relationship, the duration of the test, the slope (*b*), the index of precision (λ), and the sensitivity are recorded.

TABLE XXIX

Summary of Androgen Methods (Capon's Comb—Intramuscular Injection)

Material	Relationship	Duration of test days	Slope (*b*)	Index of precision (λ)	Sensitivity mg.	Reference
Androsterone	Log dose-comb growth	3	8.09	0.189	0.3	Greenwood *et al.* (1935)
Androsterone	Log dose-comb growth	5	12.72	0.180	0.5	
Androsterone	Log dose-log comb growth	3	0.568	0.197	0.075	Emmens (1939)
Androsterone	Log dose-log comb growth	5	0.404	0.280	0.125	
Androsterone	Log dose-comb growth	5	10.52	0.271	0.200	McCullagh and Cuyler (1939)

TABLE XXX

Summary of Androgen Methods (Capon's Comb—Inunction)

Material	Relationship	Duration of test days	Slope (*b*) ±S.E.	Index of precision (λ) ±S.E.	Sensitivity mg.	Reference
Androsterone	Log dose-comb growth	3	7.37 ±0.74	0.184 ±0.063	1.2	Emmens (1939)
Androsterone	Log dose-comb growth	5	30.3	0.076	7.0	McCullagh and Cuyler (1939)

Table XXIX deals with those methods involving administration of the hormone by injection and measuring the response by the growth of the capon's comb. The 3-day tests appear to have an accuracy equivalent to that of the 5-day tests. Thus, using the method of Greenwood *et al.* (3), the value of λ was 0.189 for the 3-day test and 0.180 for the 5-day test—the difference is not significant. Using the test of Emmens (1939), λ actually was smaller for the 3-day test. For the

5-day test the sensitivity appears to be in the range of 0.2 to 0.3 mg. androsterone.

The use of the inunction technic results in a method approximately 50 times as sensitive as the injection method (Table XXX). The precision of the local method is at least as great as the injection method, or greater. The method of McCullagh and Cuyler (1939) showed the low value of (λ) equal to 0.076. Thus, using a design consisting of 10 animals on the unknown and 10 on the standard, an accuracy of -15 to $+17$ at $P = 0.95$ could be expected.

TABLE XXXI

Summary of Androgen Methods
(Chick's Comb)

Material	Route	Relationship	Duration of test days	Slope (b) ± S.E.	Index of precision (λ) ± S.E.	Sensitivity	Reference
Testosterone propionate	Application	Log dose-comb weight	7	0.461 ± 0.035	0.289 ± 0.026	20 μg.	Dorfman (1948a) and Dorfman (1949)
Testosterone	Application	Log dose-comb weight	7	103 ± 9	0.334 ± 0.038	50 μg.	Dorfman (1949)
Testosterone propionate	Subcutaneous injection	Log dose-comb weight	5	65.9 ± 10.5	0.308 ± 0.033	500 μg.	Dorfman (1949)
Methyltestosterone	Oral	Log dose-comb weight	10	160 ± 25	0.348 ± 0.028	5 mg. Kilo Food	Dorfman (1949)
Androsterone	Application	Log dose-comb weight	7	44.1 ± 3.68	0.424 ± 0.033	15 μg.	Valle et al. (1947)
Androsterone	Application	Log dose-comb weight	7	115.1	0.244	50 μg.	

The chick comb methods, although more convenient than the capon methods, have a lower precision. These are summarized in Table XXXI.

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CONTENTS

I. Introduction

1. Physiological Action of Adrenal Cortical Steroids

The biological action of adrenal cortical steroids has been evaluated in a comprehensive review by Ingle (1944a). For the purpose of setting forth the various biological methods used for assay it may be well to review briefly the various biological end points that have been used. Adrenalectomy in experimental animals or Addison's disease in humans illustrates the relation of the adrenal cortical steroids to the body economy. Animals deprived of adrenal cortical hormones usually die within varying periods of time, depending on the species, the age at operation, the diet, and other environmental conditions. The drake will survive only a matter of hours after bilateral adrenalectomy while rats, cats, mice, and monkeys survive a number of days after operation when kept on an ordinary diet.

In a species such as the rat, if all other factors are kept constant, the survival time is dependent upon the age at which the operation is performed. This has been strikingly demonstrated in the rat itself. Twenty-day-old animals show a mean survival of 5 days, 60-day-old rats survive about 15 days, and at 360 days the mean survival is prolonged to a mean of 25 days (Grollman, 1947).

The diet, particularly the concentrations of sodium and potassium are important factors in determining the survival of adrenalectomized animals. High concentrations of dietary sodium and low concentrations of potassium favor survival whereas low concentrations of sodium and high concentrations of potassium tend to precipitate death. This is due to the fact that adrenalectomized animals tend to lose abnormal amounts of sodium in the urine while being unable to excrete potassium.

Although the life of the adrenalectomized animal may be prolonged by careful adjustment of the sodium and potassium intake, such animals still show extreme sensitivity to various forms of stress such as changes in environmental temperature, external pressure, toxins, chemical poisons, infections, and trauma. The decreased resistance to these noxious agents appears to be related to certain influences of the adrenal cortical hormones on carbohydrate metabolism, particularly the function of facilitating the conversion of proteins to carbohydrates.

Adrenal cortical steroids influence protein metabolism. When administered in relatively high concentrations these hormones cause dissolution of lymphoid tissue, a decrease in the total leucocyte count, a decrease in the absolute number of lymphocytes, and an increase in the absolute number of polymorphonuclear cells. Simultaneously there is a

rise in the total serum proteins which appears to be principally in the globulin fraction (White and Dougherty, 1946).

Other changes in the adrenalectomized animal include such circulatory changes as hemoconcentration, decreased blood pressure and blood flow. Certain digestive disturbances such as loss of appetite, poor gastro-intestinal absorption, and diarrhea also occur. The last symptom is particularly marked in advanced adrenal insufficiency.

2. Hormones of the Adrenal Cortex

All symptoms resulting from adrenal cortical insufficiency may be reversed by the administration of suitable extracts of the adrenal cortex. Six well-defined crystalline compounds having adrenal cortical activity
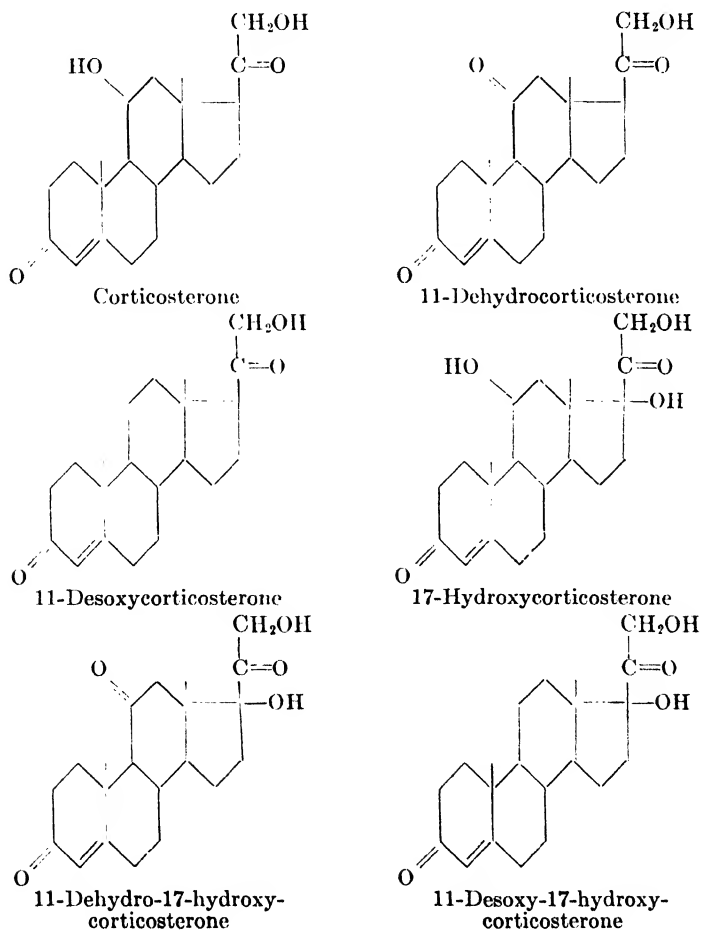
FIG. 1. Adrenal Cortical Hormones.

have been isolated and characterized (Fig. 1). Some of these compounds
are capable of correcting all the symptoms of adrenal insufficiency while
others have more limited biological activity. Four of the compounds,
corticosterone, 17-hydroxycorticosterone, 11-dehydrocorticosterone, and
11-dehydro-17-hydroxycorticosterone have oxygen at carbon atom 11,
either as a hydroxyl group or a carbonyl group, while 2 of the compounds
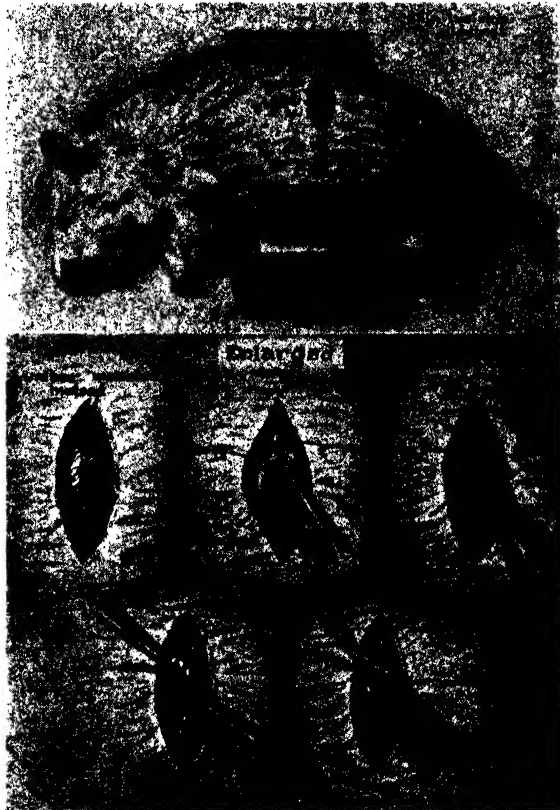
FIG. 2.   Technic of adrenalectomy in immature rat showing the position of the skin
incision (a) and the manner of extirpating the left gland (Grollman, 1941).

do not have oxygen at this nuclear position. The 11-oxygen substituted
compounds are active with respect to carbohydrate metabolism, that is,
gluconeogenesis. In addition to the 6 compounds mentioned, some
22 other adrenal cortical steroids have been isolated from this tissue.
Some of these compounds have been shown to be inactive while others
have been inadequately studied. The isolation and chemistry of the
adrenal cortical steroids have been reviewed recently (Heard, 1948).

II. Adrenalectomy

The bulk of the assay methods described in this chapter use adrenal-
ectomized rats and mice. Since similar operative procedures are
employed in both species a description of the adrenalectomy in the rat
is presented. The method is that of Grollman (1941) and is described
by reference to Figs. 2 and 3, reprinted from Grollman's publication.

The rat is anesthetized with ether, and placed upon a block 8x3x2 cm.
in size so as to elevate the viscera. A transverse incision about 5 mm.
long is made in the midline at the costovertebral angle (Fig. 2, a). The

FIG. 3. Technic of adrenalectomy in immature rat showing the manner of extirpating
the right gland (Grollman, 1941).

skin is retracted to the left, and the lumbar muscles incised just superior
and anterior to the splenic shadow (Fig. 2, b). The proper placement
of the incisions is essential, for in this way the adrenal glands appear
directly beneath the incision and require no wide dissections, search,
or exterioration of the kidney as advocated by previous authors. The
manner of grasping the peri-adrenal tissues is shown in Fig. 2 (c, d, and e).
In this way the gland or its capsule need never be touched and, as shown
in Fig. 2 (f), the intact gland together with the peri-adrenal fat and
mesenteric attachments are removed *in toto*. Adherence to the above
technic avoids leaving remnants of the capsule to which cortical tissues
are adherent, or of accessory adrenal rests which are present in about
7% of animals.

After ablation of the left adrenal the animal is turned about and the right gland is removed through the original skin incision, as shown in Fig. 3. The incision through the lumbar muscles is now made (Fig. 3, a) just above and anterior to the prominent lumbocostal artery which is seen near the costal margin, as indicated in the figure. The curved forceps are inserted over the kidney (Fig. 3, b) and by elevating the liver, which covers the adrenal on this side, the gland is brought into view and included in the forceps as shown in Fig. 3 (c); it is then removed as shown in Fig. 3 (d and e). Since the incisions made in the lumbar
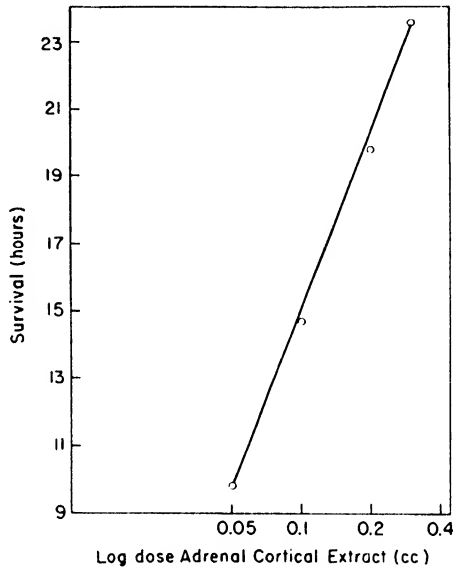
Fig. 4. Response of the adrenalectomized drake to adrenal cortical extract (Bülbring, 1937).

muscles need not exceed 3 mm. in length and may be made by spreading the blades of a pair of scissors, hemostasis or closure by sutures is unnecessary. A single suture with thread (silk or cotton) or skin clip completes the operation. The entire procedure is done in a time sufficiently short to avoid the use of non-volatile anesthetics, and it is not necessary to continue etherization during the operation. The animals appear normal in every respect within a few minutes following the operation.

III. SURVIVAL-GROWTH METHODS

1. Drake (Bülbring, 1937)

Bülbring (1937) has described a method using the bilaterally adrenalectomized drake. Drakes weighing 1.4 to 1.9 kg. are operated, using

ether-chloroform anesthesia, and immediately are injected with the test material once hourly for 20 hr. The method has not been extensively used; however, from the original data it can be seen (Fig. 4) that a linear relationship exists between the logarithm of the dose and the response using an adrenal cortical extract. The slope was 17.48 and λ, the index of precision, was found to be 0.215 ± 0.036 (Bülbring, 1937; Bliss and Cattell, 1943).

2. Rat

A. METHOD OF GROLLMAN (1941)

Young male rats at the age of 20 days were placed on the diet described in Table I for 10 days. After 10 days on this diet all animals weighing

TABLE I

Diet for Adrenalectomized Rats (Grollman, 1941)

Constituent	Amount, grams
Yellow corn meal	2280
Powdered whole milk	1000
Linseed oil meal	480
Casein	150
Alfalfa meal	60

50 g. or more were bilaterally adrenalectomized. A few hours after the operation the animals were weighed and injected subcutaneously with graded quantities of hormone or extract. Extracts were dissolved either in saline or 10% ethanol, while such steroids as desoxycorticosterone were dissolved in a mixture containing 10% ethanol, 20% propylene glycol and 80% water. On each of the 6 following days the injections were repeated and on the seventh day the final weight was recorded. The mean weight gain was calculated from the initial and final weights of the individuals in the assay group. All animals surviving more than 2 weeks after the cessation of injections were not considered in the calculations of potency. Adrenal cortical remnants and accessory tissues were found in such surviving rats. Table II (Fig. 5) illustrates the results published by Olson *et al.* (1944a) using this method for desoxycorticosterone acetate. The log dose-response relationship for this method is linear. The slope is 33.16 and λ has been estimated to be 0.154. The method is sensitive to 360 μg. of desoxycorticosterone acetate.

One difficulty of this method as pointed out by Olson *et al.* (1944a) is the fact that, at least in the dosage range studied, the slopes of adrenal cortical extract were significantly different from that found with desoxycorticosterone acetate. This may be due not so much to the difference in activity of this pure compound and the mixture in the extract as to

the fact that desoxycorticosterone was used in the form of the acetate.
Thus, the acetate had a prolonged action compared with the more fleeting
activity of the aqueous extract.

TABLE II

The Body Weight Response of Adrenalectomized Rats to Desoxycorticosterone·Acetate[a]
(Olson et al., 1944a)

Total dose of desoxycorticosterone acetate mg.	Number of rats	Mean body growth in 6 days g. ± S.E.[b]
0.24	6	All died
0.36	6	3.8 ± 2.6 (1 dead)
0.60	8	17.4 ± 2.3
0.90	9	21.6 ± 1.2
1.50	6	24.8 ± 1.8

[a] Rats injected daily for six consecutive days.
[b] S.E. Standard error of the mean.
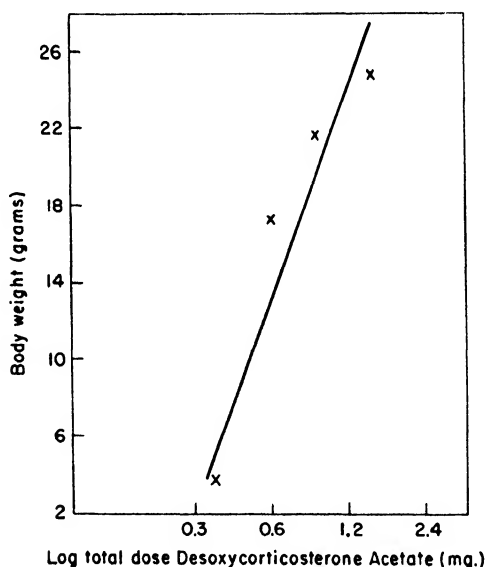
Fig. 5.   Weight response of adrenalectomized rats to desoxycorticosterone acetate
(Olson *et al.*, 1944a).

B. METHOD OF CARTLAND AND KUIZENGA (1936)

Male albino rats are bilaterally adrenalectomized at 28 days of age.
The animals are injected daily for 10 to 20 days as the case may be, for
the individual experiments.   Body weights are determined at the start

and the end of the test period, and the gain in weight calculated for each animal.

TABLE III

The Growth Response 28-Day-Old Male Adrenalectomized Rats to Adrenal Cortical Steroids[a]

Compound	Amount per day μg.	Number of rats	Mean body weight gain in 10 days g. ± S.E.
Corticosterone	50	3	4 ± 2.0
	67	5	10.0 ± 2.8
	100	15	10.5 ± 1.4
	125	9	7.8 ± 2.2
	250	10	17.7 ± 2.1
17-Hydroxycorticosterone	50	5	11.2 ± 1.1
	100	5	19.0 ± 2.2
	200	5	24.4 ± 3.8
17-Hydroxy-11-dehydrocorticosterone	67	10	12.3 ± 1.4
	156	5	16.2 ± 0.9
	312	5	22.8 ± 1.8

[a] Injected daily for 10 days.

TABLE IV

The Growth Response of 28-Day-Old Male Adrenalectomized Rats to Adrenal Cortical Steroids[a]

Compound	Amount per day μg.	Number of rats	Mean body weight gain in 10 days g. ± S.E.
Corticosterone	100	5	15.6 ± 4.4
	250	9	36.0 ± 3.2
17-Hydroxycorticosterone	50	5	15.6 ± 4.9
	100	7	31.7 ± 6.5
	200	5	41.6 ± 5.8
17-Hydroxy-11-dehydrocorticosterone	100	10	18.9 ± 3.1
	125	6	22.0 ± 4.9
	156	5	25.8 ± 2.2
	312	5	33.6 ± 2.5

[a] Injected daily for 20 days.

Tables III and IV illustrate the growth response of adrenalectomized rats to varying doses of corticosterone, 17-hydroxycorticosterone, and 11-dehydro-17-hydroxycorticosterone during 10- and 20-day test periods respectively (Kuizenga, 1949). The relationship of log dose to response

was found to be linear in each case (Fig. 6). Table V gives the slopes of the three steroids in the 10-day test. The slopes for corticosterone, 17-hydroxycorticosterone, and 11-dehydro-17-hydroxycorticosterone were
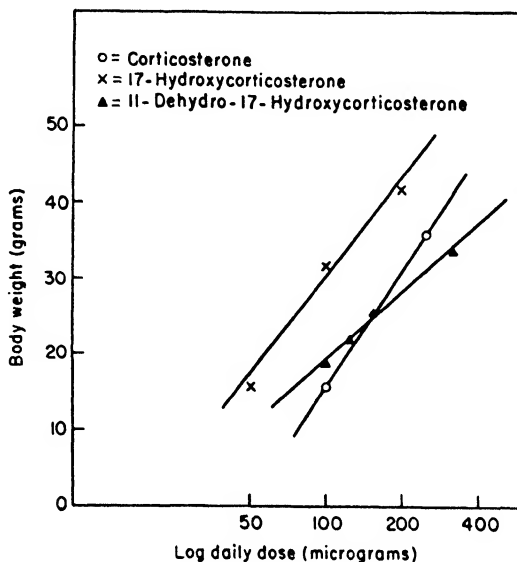
FIG. 6. Weight response of adrenalectomized rats to adrenal cortical steroids (Kuizenga, 1949).

21.19, 21.96, and 13.21 respectively. Also tabulated is λ, the index of precision. These values varied from 0.246 to 0.287. Specifically, the use of 20 animals on the unknown run simultaneously with 20 animals

TABLE V

The Precision of the Growth Method of Assay[a]

Compound	Slope, b	Index of precision λ
Corticosterone	21.19	0.279
17-Hydroxycorticosterone	21.96	0.246
17-Hydroxy-11-dehydrocorticosterone	13.21	0.287

[a] Injected daily for 10 days.

TABLE VI

The Growth Response of 28-Day-Old Male Adrenalectomized Rats to Adrenal Cortical Steroids[a]

Compound	Slope, b	Index of precision λ
Corticosterone	49.63	0.195
17-Hydroxycorticosterone	43.20	0.328
17-Hydroxy-11-dehydrocorticosterone	28.15	0.309

[a] Injected daily for 20 days.

on the standard will result in a determination of the potency ratio with an error range of -33 to $+48$ at $P = 0.95$.

Table VI gives the slopes and indices of precision for the 20-day tests. Here slopes of 49.63, 43.20, and 28.15 respectively were found for corticosterone, 17-hydroxycorticosterone, and 11-dehydro-17-hydroxycorticosterone. The indices of precision varied from 0.195 to 0.328.

Table VII summarizes the relative activity of the three steroids by the 20-day growth test. 11-Dehydro-17-hydroxycorticosterone was chosen as the standard. No departure from parallelism was found, with t values of 0.365 and 1.263 for the studies on 17-hydroxycorticosterone and corticosterone respectively.

TABLE VII

The Relative Potency of Adrenal Cortical Steroids and an Adrenal Extract on the Basis of a Growth Method[a] (Cartland and Kuizenga, 1936)

Material	Number of animals	Slope, b	Relative potency	Error range $(P = 0.95)$ %	t
17-Hydroxy-11-dehydrocorticosterone	26	28.2	100	. . . .	. . . . .
17-Hydroxycorticosterone	18	43.2	219	$-34; +51$	0.365
Corticosterone	14	49.6	108	$-32; +47$	1.263

[a] Twenty-day test.

3. Mouse (Dorfman, 1949b)

Male Swiss mice (6–9 g.) are bilaterally adrenalectomized under ether anesthesia. Animals subjected to excessive trauma usually die within 24 hr. Those alive the day following the operation are injected subcutaneously with the hormone dissolved in 0.05 ml. of corn oil. The injections are continued for 10 successive days. The diet consisted of

TABLE VIII

The Response of the Male Immature Adrenalectomized Mouse to Desoxycorticosterone Acetate[a]

Daily dose of desoxycorticosterone acetate μg.	Total number of animals	Number alive on the 10th day	Per cent of animals alive
0	36	29	19
12.5	20	14	30
25	25	14	44
50	37	22	41
100	36	7	81
400	10	8	80

[a] Mice injected daily for 10 days.

commercial dog chow (Purina) with no supplementation and tap water to drink.

Table VIII illustrates the response to 12.5, 25, 50, 100, and 400 μg. of desoxycorticosterone acetate. Increasing the dosage from 100 to 400 μg. produced no further increase in the percentage of surviving animals. Extensive critical studies of this method are yet to be made (Dorfman, 1949b).

IV. Electrolyte Methods

1. Sodium Methods

The influence of adrenal cortical steroids and extracts on sodium metabolism has been studied in dogs and rats with the view of utilizing the change as an indication of adrenal cortical activity. Such an assay on crude material is complicated by the fact that not all biologically adrenal cortical steroids possess the same qualitative influence on sodium metabolism. Thus desoxycorticosterone leads to sodium retention in both adrenalectomized and normal animals while such compounds as 17-hydroxycorticosterone cause sodium excretion even in adrenalectomized animals. Thus it is possible that a mixture of desoxycorticosterone and 17-hydroxycorticosterone will not change the urinary sodium concentration due to the balancing of the sodium retaining and excreting influences of the respective compounds. This difficulty must be borne in mind when utilizing such a test as the sodium excretion test. Although this type of adrenal test has been employed for some 15 years, no exact definition of errors is possible at this time for either the dog or rat methods described below. Some idea of the sensitivity is indicated.

A. DOG (HARTMAN *et al.*, 1940)

This method is based on the work of Hartman *et al.* (1940, 1941) as applied by Olson *et al.* (1944a).

Both male and female dogs ranging in weight from 8–15 kg. were used. After preliminary control experiments, testing their constancy and sensitivity in the assay, 4 males and 3 females were selected from a group of 10 males and 10 females. The males were segregated from the females to avoid excitement. The females were fed the diet of Hartman *et al.* (1941) which for a 10- to 12-kg. dog consisted of 250 g. of beef heart, 70 g. of Purina dog chow, 1 yeast pill, and 2 g. of sodium chloride daily. Cod liver oil capsules were given once a week. The males were fed Purina dog chow exclusively.

Nine hours before the test, usually at 11 P.M., the dogs were fed. At the start of the experiment the animals were catheterized, given

100 ml. of water, and placed in metabolism cages for a 6-hr. period for the quantitative collection of the urine.   At the end of the 6-hr. period, the animals were again catheterized.   Desoxycorticosterone acetate in a solution containing 10% ethanol, 10% propylene glycol, and 80% water was employed as the standard.   The solvent was found to exert no influence upon sodium excretion.   Urinary sodium was determined by the method of Butler and Tuthill (1931).

A standard dose of 0.7 mg. of desoxycorticosterone acetate was employed and the unknown assayed in comparison 2 days later if the decrease in sodium in the standard run lay between 35 and 65% of the control level.   Typical results are presented in Table IX.   A unit was defined by the following relationship:

$$\text{No. of units} = \frac{\% \text{ Retention by unknown}}{\% \text{ Retention by 0.7 mg. desoxycorticosterone}} \times 10$$

TABLE IX

The Influence of Desoxycorticosterone Acetate[a] on the Sodium Excretion in Adrenalectomized Rats (Olson et al., 1944a)

Dog	Number of tests	Decrease in sodium excretion %' + S.E.	Season
LBM	4	40.1 ± 4.1	Summer
LBM	5	45.7 ± 2.6	Winter
BM		48.4 ± 3.1	Summer
BM		51.7 ± 3.6	Winter
Composite		51.0 ± 1.4	Summer and winter

  [a] Dosage = 0.7 mg.

B. RAT (DORFMAN et al., 1947)

Recently a sodium method has been suggested which utilizes radio-sodium and which shows promise as a quantitative method (Dorfman et al., 1947).   The method has a high sensitivity.   The reproducibility will depend upon how well conditions can be controlled in actual practice.

Male albino rats between 100 and 175 g. in weight were bilaterally adrenalectomized in one stage under ether anesthesia.   The animals were fed a diet of Purina dog chow and tap water.   The day following the operation the animals received the test dose of material dissolved in 0.25 ml. of corn oil.   All food was removed from the cages.

One hour after the administration of the test material the radio-sodium as sodium chloride was administered in 2 ml. of solution.   The total sodium chloride was 35 μg. per gram of body weight.   Since adrenalectomized rats under the conditions of the experiment excrete about 2% of the administered radiosodium, and since it is desirable to have counts in the region of 1000 counts per minute it is advisable to administer between 50,000 to 100,000 counts per minute of radiosodium.

After receiving the radiosodium the animals were immediately placed
in metabolism cages and the urine collected quantitatively for a period
of 6 hr.

The urine was collected directly in a 2-oz. ointment capsule, and the
cage was washed with three portions of a 0.1% sodium chloride solution.
The urine plus the three 10 ml. washings contained in the ointment
capsule were evaporated to dryness by overhead heating with a 300-w.
infrared lamp to avoid bumping and sputtering. The radiosodium
contained in the dried residue was counted with a suitable instrument,
and the amount excreted was expressed in per cent of the administered
material.

TABLE X

The Relative Activity of Various Steroids on a Sodium Excretion Test in Adrenalectomized
Rats (Dorfman, 1949b)

Compound	Dosage µg.	Type of activity
Desoxycorticosterone	1	Retention
Desoxycorticosterone acetate	10	Retention
Δ^4-Pregnenol-21-trione-3,12,20-21-acetate	100	Negative
	400	Negative
	1340	Retention
17-Hydroxycorticosterone	25	Negative
	50	Increased excretion
Testosterone	2000	Negative
α-Estradiol	2000	Negative

Table X is a summary of experience with this method. As little as
1 µg. of desoxycorticosterone and 10 µg. of the acetate produced sig-
nificant retention of sodium. The Δ^4-pregnenol-21-trione-3,12,20-21-
acetate was active at 1340 µg. 17-Hydroxycorticosterone at 50 µg.
showed increased sodium excretion. Testosterone and α-estradiol at
2000 µg. were inactive.

2. Potassium Methods

Two methods are described in this section, one using normal mice,
the other, adrenalectomized rats.

A. MOUSE (TRUSZKOWSKI AND DUSZYNSKA, 1940)

Normal male mice, 28 days of age and weighing 7–9 g., are injected
subcutaneously with 0.1 ml. of an oil solution of hormone at 1 P.M. At
9 A.M. the following day the animals receive a second injection of hormone.
Four hours after the second injection, 0.3 ml. of a 2.5% solution of KCl
is injected intraperitoneally and the results evaluated on the per cent of
animals surviving.

The influence of desoxycorticosterone acetate is presented in Table

XI from the work of Truszkowski and Duszynska (1940). Between the dosages of 125 to 500 μg. of the hormone a linear relationship was found with a slope of 94.86 and a value of 0.086 for λ. This would indicate an exceedingly high order of reliability.

TABLE XI

The Influence of Desoxycorticosterone Acetate on a Potassium Intoxication Test
(Truszkowski and Duszynska, 1940)

Total dose mg./10 g. B.W.	Number of animals	Deaths %	Survival %	Standard deviation
0	39	66.6	33.4	7.5
0.125	10	70.0	30.0	14.5
0.250	25	52.0	48.0	10.0
0.500	55	32.7	67.3	6.3
1.000	28	35.7	64.3	9.0
2.000	30	33.3	66.7	8.6

B. RAT (DORFMAN, 1949a)

Albino rats weighing between 40 and 50 g. are bilaterally adrenal-ectomized and placed on a stock diet such as Purina dog chow and tap water. The hormone is administered 48, 51, and 52½ hr. after operation. The material is dissolved in a 10% ethanol-90% water solution and administered by stomach tube so that each dose is contained in 1 ml. of solution. If the material is to be injected, the total dose may be administered 48 hr. after adrenalectomy. Four hours after the first oral administration or the injection, as the case may be, the potassium chloride is administered intraperitoneally as a 3.3% solution. About 0.4 to 0.5 mg./g. of body weight of potassium chloride is administered. This quantity of potassium chloride will kill 80 to 100% of the animals within 2 hr. It is desirable to make a preliminary run on a small group of operated animals to ascertain the optimum dose of potassium chloride. A minimum of 10 rats per group should be employed with one group of solvent-treated controls run simultaneously.

A radiopotassium method similar to the radiosodium technic previously described for the detection of minute amounts of desoxycorticosterone has been investigated. At the present time only a statement with respect to sensitivity can be made. As little as 10 μg. of desoxycorticosterone can be detected by the radiopotassium method using adrenalectomized rats (Dorfman, 1949b).

Tables XII and XIII present typical results using adrenal cortical extracts and desoxycorticosterone on the rat potassium intoxication

test (Feil and Dorfman, 1945). This needs further study to evaluate its quantitative usefulness.

TABLE XII

The Influence of Desoxycorticosterone Acetate on Potassium Intoxication of the Adrenalectomized Male Rat (Feil and Dorfman, 1945)

Amount of KCl administered orally	10 % ethanol-treated rats			Desoxycorticosterone acetate-treated rats			
mg./g. B.W.	No. of rats	Mean B.W. g.	% Dead	No. of rats	Mean B.W. g.	Total amt. DCA mg.	% Dead
0.50	10	43	100	10	47	0.75	70
0.45	14	42	57	8	42	0.75	0
0.45	16	44	100	10	46	1.50	50
0.45	14	42	57	10	38	1.50	20
0.45	14	42	57	10	45	2.25	20
0.45	14	42	57	9	47	2.25	33

TABLE XIII

The Influence of Adrenal Cortical Extract on Potassium Intoxication of Adrenalectomized Male Rats (Feil and Dorfman, 1945)

Amount of KCl administered orally	10% ethanol-treated rats			Adrenal cortical extract-treated rats			
mg./g. B.W.	No. of rats	Mean B.W. g.	% Dead	No. of rats	Mean B.W. g.	Total volume extract ml.	% Dead
0.45	14	42	57	10	42	0.15	0
0.50	10	43	100	10	42	0.30	30
0.45	16	44	100	10	43	0.30	0
0.35	12	44	75	12	46	0.60	0
0.45	14	42	57	10	43	0.60	0

V. CARBOHYDRATE METHODS

Four types of carbohydrate methods have been studied; three in great detail. The rat glycogen, mouse glycogen, and muscle-work tests have been evaluated carefully and will be presented in detail. The

fourth method which is concerned with the anti-insulin action of certain adrenal cortical hormones will be mentioned briefly. The rat glycogen method for the assay of adrenal cortical hormones was suggested originally by Reinecke and Kendall (1942). These workers set up the conditions for the assay. From a limited set of data the value of λ was calculated and found to be 0.168 with a slope of 59.6.

1. Rat Glycogen Methods

A. METHOD OF OLSEN *et al.* (1944a)

Male albino rats, 60–75 days of age and weighing 145–185 g. are bilaterally adrenalectomized under ether anesthesia (see p. 329). They are placed on the high protein diet described in Table XIV and are given 1% sodium chloride in their drinking water until the morning of the fourth postoperative day. The animals are fasted for 24 hr. until the morning of the fifth postoperative day. The drinking water is removed and injections are started. The hormone in saline or 10%

TABLE XIV

Diet for Adrenalectomized Rats (Olson et al., 1944a)

Constituent	%
Casein	58.0
Dried brewer's yeast	6.0
Sucrose	8.5
Lard	19.0
Hawk-Oser (1931) salt mixture	4.0
Calcium carbonate	1.0
Cod-liver oil	1.0
Cereal cellulose	2.0
Cystine	0.3
Choline chloride	0.2
Total	100.0

ethanol is administered in 4 equally divided doses at 2-hr. intervals. Two hours after the last injection, 1.5 ml. of a 1% solution of sodium amytal is injected intraperitoneally. One half of the left lateral lobe of each liver is removed and dropped into a tared, tapered 50 ml. centrifuge tube containing 2.0 ml. of cold 30% KOH (Cori, 1932). The samples are quickly weighed, placed in a boiling-water bath, and the tissue is digested. The glycogen is isolated and hydrolyzed by the method of Good *et al.* (1933). The reducing substances are determined with reagent 50 by the method of Shaffer and Somogyi (1933).

Table XV lists the influence of various steroids on the deposition of liver glycogen in the adrenalectomized rat. The data are those of

Olson *et al.* (1944b). The 4 steroids studied were corticosterone, 11-dehydrocorticosterone, 17-hydroxycorticosterone, and 11-dehydro-17-hydroxycorticosterone. Table XVI lists the linear regression coefficients for the 4 steroids which influence glycogen deposition. On the basis of

TABLE XV

Glycogen Deposition in Adrenalectomized Rats (Olson et al., 1944b)

Steroid	Total dose mg.	Number of rats	Mean liver glycogen deposition % ± S.E.
Corticosterone	0.39	4	0.44 ± 0.08
	0.47	4	0.52 ± 0.10
	0.58	4	0.84 ± 0.06
	0.62	3	0.72 ± 0.11
	0.94	4	1.15 ± 0.09
	1.15	4	1.40 ± 0.17
	1.24	4	1.39 ± 0.19
11-Dehydrocorticosterone	0.39	5	0.66 ± 0.09
	0.42	5	0.47 ± 0.05
	0 79	6	1.00 ± 0.09
	0.87	9	1.29 ± 0.08
	1.16	5	1.40 ± 0.18
	1.74	7	1.98 ± 0.16
11-Dehydro-17-hydroxycorticosterone	0.33	4	0.79 ± 0.13
	0.39	6	0.61 ± 0.12
	0.39	5	0.61 ± 0.08
	0.66	5	1.10 ± 0.15
	0.77	6	1.05 ± 0.10
	0.79	5	1.31 ± 0.13
	1.27	5	1.72 ± 0.16
17-Hydroxycorticosterone	0.44	4	0.73 ± 0.07
	0.54	4	1.22 ± 0.05
	0.72	4	1.28 ± 0.14
	0.88	4	1.80 ± 0.10
	0.93	6	1.85 ± 0.16
	1.08	4	2.09 ± 0.17

TABLE XVI

The Regression Coefficients for Various Adrenal Cortical Steroids (Olson et al., 1944b)

Steroid	a ± S.E.	b ± S.E.	t
Corticosterone	1.24 ± 0.05	2.06 ± 0.29	
11-Dehydrocorticosterone	1.37 ± 0.05	2.14 ± 0.21	0.23
11-Dehydro-17-hydroxycorticosterone	1.46 ± 0.05	1.91 ± 0.27	0.37
17-Hydroxycorticosterone	1.95 ± 0.05	3.29 ± 0.41	2.45

these data the relative activities of the various steroids were calculated and are presented in Table XVII. 11-Dehydro-17-hydroxycorticosterone was chosen as the standard. On this basis corticosterone was found to be 75% as active as the standard, and 11-dehydrocorticosterone had a relative activity of 88%. The fourth steroid, 17-hydroxycorticosterone, was found to have a slope significantly greater than the standard ($t = 2.45$), and therefore, the relative potency was not calculated. Olson *et al.* (1944a) have used this method to assay the potency of adrenal cortical extracts in terms of corticosterone. Five such extracts had slopes not significantly different from the standard, corticosterone.

Table XVII and XVIII illustrate the precision of the Olson *et al.* (1944a) method for the various steroids studied. The index of precision,

TABLE XVII

The Relative Activities of Adrenal Cortical Compounds Expressed in Terms of 11-Dehydro-17-hydroxycorticosterone (Olson et al., 1944b)

Compound	Number of animals	Slope b	Potency ratio %	Error ($P = 0.95$) %
11-Dehydro-17-hydroxycorticosterone	36	1.82	100	. . . .
Corticosterone	23	1.88	75	−16 to +20
17-Hydroxycorticosterone	26	3.20	. . .	. . . .
11-Dehydrocorticosterone	37	2.05	88	−13 to +16

TABLE XVIII

The Precision of the Olson et al. (1944a, 1944b) Method

Compound	Slope b	Index of precision λ	Sensitivity μg.
Corticosterone	2.06	0.112	300
11-Dehydrocorticosterone	2.14	0.126	300
11-Dehydro-17-hydroxycorticosterone	1.91	0.141	300
17-Hydroxycorticosterone	3.29	0.077	300

λ, varied from 0.077 to 0.141. If we consider the mean value of λ, we find that the use of 10 animals on the unknown and 10 on the standard should result in an accuracy of −21% to +26% at $P = 0.95$ for the determination of the potency ratio. When the number of animals is doubled, that is the use of a total of 40 animals, the error range at $P = 0.95$ is −15% to +18%.

B. METHOD OF PABST, SHEPPARD, AND KUIZENGA (1947)

The method is based on the ability of certain adrenal cortical hormones to cause glycogen deposition in the fasting adrenalectomized male

albino rat and is based on the work of Olson *et al.* (1944a, 1944b). The actual data utilized in this assay are those of the latter workers who made them available to the author for this evaluation.

Male albino rats of the Sprague-Dawley strain weighing 140–160 g. were maintained on a Purina dog chow diet. They were bilaterally adrenalectomized under ether anesthesia. After the operation the

TABLE XIX

Glycogen Deposition in the Fasting Adrenalectomized Male Rat Treated with Adrenal Cortical Hormones[a] (Pabst et al., 1947)

Hormone	Dose mg.	Liver glycogen % ± S.E.	S.D.
Corticosterone	0.35	0.45 ± 0.07	0.21
	0.50	0.87 ± 0.08	0.26
	0.71	0.95 ± 0.09	0.30
	1.00	1.28 ± 0.07	0.23
11-Dehydrocorticosterone	0.35	0.25 ± 0.03	0.08
	0.50	0.48 ± 0.05	0.17
	0.71	0.87 ± 0.09	0.29
	1.00	1.34 ± 0.08	0.26
17-Hydroxycorticosterone	0.125[b]	0.45 ± 0.07	0.20
	0.250	0.98 ± 0.09	0.27
	0.350	1.25 ± 0.08	0.27
	0.500	1.68 ± 0.08	0.25
	0.710	1.82 ± 0.11	0.35
	1.000	2.22 ± 0.14	0.43
11-Dehydro-17-hydroxycorticosterone	0.25	0.77 ± 0.07	0.22
	0.35	0.71 ± 0.09	0.29
	0.50	1.37 ± 0.07	0.21
	0.71	1.54 ± 0.10	0.32
	1.00	1.74 ± 0.06	0.20

[a] Ten animals per group.
[b] Nine animals in this group.

animals were placed on a high protein diet (see Table XIV). Tap water containing 1% sodium chloride was used as drinking water. After a 24-hr. fast, between the fourth and fifth postoperative days, the crystalline hormones dissolved in cottonseed oil were injected subcutaneously in 4 divided doses at 2-hr. intervals. The volume of oil varied from 0.18 to 0.25 ml. per injection. Between 1½ and 2½ hr. after the last injection the animals were anesthetized with 0.75 ml. of a 2% solution of cyclopal sodium administered intraperitoneally. The entire liver was removed for analysis of the glycogen content.

The liver was blotted on absorbent paper to remove the surface

blood and immediately placed in 30% KOH. Approximately 2 ml. of the potassium hydroxide solution was used for each g. of liver. Complete digestion of the liver was accomplished by heating for 30 to 40 min. on the steam bath. The method of Good *et al.* (1933) with modifications was employed. Absolute alcohol was used for precipitation of the glycogen. Hydrolysis of the glycogen was accomplished by adding 2.5 ml. of normal H_2SO_4 and autoclaving for 15 min. at 15 lb. Glucose deter-

A = o = Corticosterone
B = x = 17-Hydroxycorticosterone
C = ▲ = 11-Dehydro-17-Hydroxycorticosterone
D = ● = 11-Dehydrocorticosterone
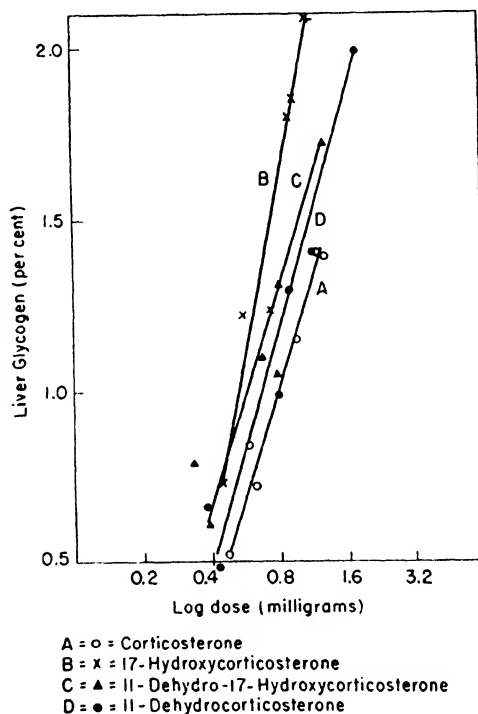
FIG. 7. Liver glycogen response of adrenalectomized rats to adrenal cortical steroids (Pabst *et al.*, 1947).

minations were done by the modified iodometric copper method of Shaffer and Somogyi (1933), using reagent 50 containing no KI. Results in Table XIX express the liver glycogen content in glucose equivalents in per cent.

Figure 7 illustrates the linear relationship which exists between the logarithm of the dose and the response using the deposition of liver glycogen in the fasting adrenalectomized male rat as the end point.

Table XX presents the relative potency of 4 adrenal cortical steroids using the method of Pabst *et al.* (1947). 11-Dehydro-17-hydroxycorticosterone was chosen as the standard and no departure from paral-

lelism of slope was found for the 4 steroids studied.   Corticosterone and 11-dehydrocorticosterone were 54 and 48% as active as the standard, whereas 17-hydroxycorticosterone had a relative potency of 155% of the standard.

TABLE XX

The Relative Activities of Various Adrenal Cortical Steroids Expressed in Terms of the Activity of 17-Hydroxy-11-dehydrocorticosterone (Pabst et al., 1947)

Compound	Number of rats	$b \pm$ S.E.	Potency ratio	Error $(P = 0.95)$ %	t
17-Hydroxy-11-dehydro-corticosterone	50	1.848 ± 0.191	100	. . . .	. . . . .
Corticosterone	40	1.677 ± 0.183	54	$-13; +15$	0.602
11-Dehydrocorticosterone	40	2.229 ± 0.251	48	$-10; +12$	1.202
17-Hydroxycorticosterone	59	1.963 ± 0.131	155	$-12; +14$	0.005

The method of Pabst *et al.* (1947) was analyzed by the 2x2 design of Bliss (1944a, 1944b). This can be employed since the following conditions hold:

1. The response is a linear function of the logarithm of the dose.

2. Two concentrations of unknown, and two of the standard are employed so that the following relationship is established.

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

When these conditions are met the calculations for slope (b), index of precision (λ), potency ratio, and error range of the potency ratio are simplified. The parallelism of the slopes of unknown and standard are measured by t.

TABLE XXI

The Assay of 11-Dehydrocorticosterone

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown
$N = 5$

Dosage levels µg.	b	λ	t	Potency ratio $\pm$ S.E.
1000; 500	286	0.088	0.934	93 ± 9
710; 500	256	0.096	0.625	107 ± 11
1000; 710	318	0.089	1.350	98 ± 9
710; 350	203	0.106	0.935	107 ± 12
500; 350	151	0.095	0.328	99 ± 10
1000; 350	217	0.093	0.906	91 ± 9

Tables XXI through XXIV illustrate the use of this simplified design when a total of 20 animals, 10 on the unknown and 10 on the standard, was employed. In each case the unknown and standard were the same and the theoretical potency ratio was 100%. The ratio of the high dose to low dose varied. When 11-dehydrocorticosterone was employed in 6 trials the potency ratio found varied from 93 to 107%. In no instance

TABLE XXII

The Assay of 11-Dehydro-17-hydroxycorticosterone

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown
$N = 5$

Dosage levels μg.	b	λ	t	Potency ratio ±S.E.
1000; 710	116	0.209	1.090	84 ± 19
1000; 500	123	0.158	0.598	110 ± 16
1000; 350	227	0.090	1.125	93 ± 9
710; 500	111	0.281	1.039	87 ± 27
710; 350	270	0.108	0.122	85 ± 10
500; 350	430	0.046	1.468	103 ± 5

TABLE XXIII

The Assay of Corticosterone

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown
$N = 5$

Dosage levels μg.	b	λ	t	Potency ratio ±S.E.
1000; 710	226	0.108	0.650	110 ± 11
1000; 500	139	0.163	0.395	103 ± 17
1000; 350	183	0.239	0.153	109 ± 25
710; 500	54	0.355	1.320	125 ± 51
710; 350	167	0.136	0.430	118 ± 16
500; 350	273	0.084	0.684	100 ± 9

was a significant difference in slopes of unknown and standard found. The standard error of the potency ratio varied from ±9 to ±12%. These data are presented in Table XXI.

Tables XXII and XXIII illustrate the use of a total of 20 animals, using the hormones 11-dehydro-17-hydroxycorticosterone and corticosterone respectively. No significant differences in slopes were found in either case. In the studies using 11-dehydro-17-hydroxycorticosterone

potency ratios of 84 to 110% were found, as compared to the theoretical value of 100%. The standard error of the potency ratio varied from ± 5 to $\pm 27\%$ in 6 trials. With corticosterone (Table XXIII) the potency ratio varied from 100 to 125%. In 5 of the 6 trials the standard error of the potency ratio varied from ± 9 to $\pm 25\%$ but in one trial the value was $\pm 51\%$.

17-Hydroxycorticosterone was studied in a similar manner over the dosage ranges of 125 to 1000 μg. per animal (Table XXIV). In 5 of the

TABLE XXIV

The Assay of 17-Hydroxycorticosterone

$$\frac{\text{High dose of standard}}{\text{Low dose of standard}} = \frac{\text{High dose of unknown}}{\text{Low dose of unknown}}$$

Standard = Unknown
$N = 5$

Dosage levels μg.	b	λ	t	Potency ratio $\pm$ S.E.
1000; 710	271	0.103	2.240	125 $\pm$ 16
1000; 500	180	0.151	1.388	159 $\pm$ 38
1000; 350	214	0.128	3.520	112 $\pm$ 10
1000; 250	207	0.142	3.195	144 $\pm$ 22
1000; 125	193	0.163	1.965	137 $\pm$ 23
710; 250	208	0.136	0.519	105 $\pm$ 15
710; 500	100	0.302	0.829	124 $\pm$ 44
710; 350	215	0.139	1.122	84 $\pm$ 12
500; 250	235	0.118	0.368	117 $\pm$ 15
500; 350	282	0.082	2.550	96 $\pm$ 8
500; 125	204	0.102	1.158	111 $\pm$ 11
350; 250	184	0.120	2.190	83 $\pm$ 11
350; 125	177	0.131	1.482	85 $\pm$ 12
250; 125	174	0.142	1.248	107 $\pm$ 16

14 trials significant differences in slopes of unknown and standard were found. In 5 trials over the dosage range of 125 to 500 μg. per animal, the potency ratio varied from 83 to 117%. The standard error of the potency ratio varied from ± 8 to $\pm 15\%$. In 8 trials, when the dosage levels of 710 and 1000 μg. were employed for either the high dose, low dose, or both, the potency ratio varied from 84 to 159%, and the standard error of the potency ratio varied from ± 10 to $\pm 44\%$. Thus, the more reliable assay range appeared to be between 125 and 500 μg.

In Tables XXV and XXVI the method has been used in another way. The relative potencies of 17-hydroxycorticosterone and 11-dehydro-17-hydroxycorticosterone are studied in Table XXV. Whereas Pabst *et al.* (1947) have found a value of 68% for 11-dehydro-17-hydroxycorticos-

terone as compared to 17-hydroxycorticosterone, the mean potency ratio for the 5 trials is 63% with a variation from 52 to 77% in individual trials when N equals 10.

Table XXVI illustrates 6 trials where the relative activities of 11-dehydrocorticosterone and 17-hydroxycorticosterone were compared,

TABLE XXV

Assay of Adrenal Cortical Steroids
Standard = 17-Hydroxycorticosterone
Unknown = 11-Dehydro-17-hydroxycorticosterone
$N = 10$

Dosage levels of unknown and standard, μg.	b	λ	t	Potency ratio $\pm$ S.E.
1000; 710	203	0.183	0.851	65 $\pm$ 14
1000; 500	152	0.167	1.062	55 $\pm$ 9
1000; 350	219	0.156	0.264	59 $\pm$ 11
710; 500	101	0.290	0.158	52 $\pm$ 23
710; 350	230	0.132	1.338	67 $\pm$ 7
500; 350	353	0.066	1.542	77 $\pm$ 5

TABLE XXVI

Assay of Adrenal Cortical Steroids
Standard = 17-Hydroxycorticosterone
Unknown = 11-Dehydrocorticosterone
$N = 10$

Dosage levels of unknown and standard, μg.	b	λ	t	Potency ratio $\pm$ S.E.
1000; 710	294	0.089	0.331	48 $\pm$ 7
1000; 500	233	0.129	1.679	36 $\pm$ 6
1000; 350	227	0.134	0.604	39 $\pm$ 5
710; 500	176	0.150	1.492	24 $\pm$ 11
710; 350	200	0.150	0.256	33 $\pm$ 7
500; 350	223	0.100	1.410	31 $\pm$ 8

the latter being considered the standard. Under these conditions 11-dehydrocorticosterone was found to be between 24 to 48% as active as the standard with a mean potency ratio of 35%. Pabst *et al.* (1947) had reported 32%.

Five comparisons of the relative activities of 11-dehydro-17-hydroxy-corticosterone and corticosterone are presented in Table XXVII. The

TABLE XXVII

Assay of Adrenal Cortical Steroids
Standard = 11-Dehydro-17-hydroxycorticosterone
Unknown = Corticosterone

Dosage levels of unknown and standard, μg.	b	λ	t	Potency ratio ±S.E.
1000; 710	181	0.152	0.774	51 ± 12
1000; 500	131	0.178	0.310	43 ± 9
1000; 350	204	0.122	1.245	67 ± 6
710; 350	222	0.139	1.715	64 ± 8
500; 350	350	0.071	1.539	78 ± 5

TABLE XXVIII

Assay of Adrenal Cortical Steroids
Standard = 17-Hydroxycorticosterone
Unknown = Corticosterone

Dosage levels of unknown and standard, μg.	b	λ	t	Potency ratio ±S.E.
1000; 710	249	0.125	0.331	44 ± 11
1000; 500	159	0.240	0.519	30 ± 10
1000; 350	199	0.160	0.700	37 ± 6
710; 350	176	0.172	0.388	34 ± 8
500; 350	285	0.097	0.090	51 ± 8

TABLE XXIX

The Precision of the Method of Pabst et al. (1947)

Compound	Slope b	Index of precision λ	Sensitivity μg.
11-Dehydrocorticosterone	239 ± 25	0.095 ± 0.002	350
11-Dehydro-17-hydroxycorticosterone	213 ± 51	0.149 ± 0.031	350
Corticosterone	198 ± 23	0.146 ± 0.024	125
17-Hydroxycorticosterone	203 ± 12	0.140 ± 0.014	125

former steroid served as the standard. Under these conditions the mean relative potency of corticosterone was found to be 61% with a variation from 43 to 78%. Pabst *et al.* (1947) had reported 48%.

Table XXVIII deals with the last study in this series in which the unknown corticosterone was compared to the standard 17-hydroxycorticosterone. The mean potency ratio was 39% as compared to the

value of 35% reported by Pabst *et al.* (1947). The 5 individual trials varied from 30 to 51%.

Table XXIX summarizes the data on the precision of the Pabst *et al.* (1947) method. The indices of precision varied from 0.095 ± 0.002 to 0.149 ± 0.031. Thus, the method has a precision similar to that found for the Olson *et al.* (1944b) method.

2. Mouse Glycogen Methods

Three groups of workers have studied the liver glycogen response of the adrenalectomized mouse to adrenal cortical steroids. (Venning *et al.* (1946), Eggleston *et al.* (1946), and Dorfman *et al.* (1946).) The methods are essentially of two types. The first, such as that of Venning *et al.* (1946), depends upon the deposition of glycogen in the fasting adrenalectomized mouse in the depleted liver. The methods employed by Eggleston *et al.* (1946) and Dorfman *et al.* (1946) use the fasting adrenalectomized mouse and depend on protecting the fall in liver glycogen. Examples of these two methods will be discussed in detail.

A. METHOD OF VENNING *et al.* (1946)

It is important to use a single strain of mice. Two days before adrenalectomy the mice are placed on the McCollum diet (Table XXX)

TABLE XXX

McCollum Diet Used in the Mouse Glycogen Methods

Constituent	%
Whole wheat flour	67.5
Casein	15.0
Whole milk powder	10.0
Butter	5.0
Calcium carbonate	1.5
Sodium chloride	1.0

which contains 26% protein and 52% carbohydrate. The male mice (20–25 g.) are bilaterally adrenalectomized and kept at a constant temperature of 76°F. The animals receive a solution of 0.9% NaCl containing 5% glucose for drinking water on the first postoperative day. On the morning of the second postoperative day, 0.9% NaCl solution is used for drinking water. At 5:00 P.M. on the third postoperative day, the food is removed. On the fourth postoperative day, beginning at 9:15 A.M. a total of 7 subcutaneous injections are given at 9:15 A.M., 10:00 A.M., 10:45 A.M., 11:30 A.M., 12:30 A.M., 1:30 P.M., and 2:30 P.M. The total volume injected is 1.4 ml. The solution consists of the hormone in 10% ethanol to which 70 mg. of glucose has been added for each 1.4 ml. of solution. At 3:30 P.M., 1 hr. after the last injection, the animals

TABLE XXXI

The Influence of Adrenal Cortical Steroids on the Deposition of Liver Glycogen in the Fasting Adrenalectomized Mouse (Method of Venning et al., 1946)

Compound	Total amount administered μg.	Number of mice	Mean liver glycogen mg./100 g. ± S.E.
0	0	34	4.1 ± 0.5
11-Dehydrocorticosterone	40	8	42 ± 4.8
	80	8	69 ± 7.9
	160	9	94 ± 7.1
11-Dehydro-17-hydroxycorticosterone	10	8	33 ± 3.2
	10	8	35 ± 2.5
	20	8	61 ± 2.0
	20	8	63 ± 4.4
	40	8	91 ± 7.5
	50	8	102 ± 7.8

are weighed and anesthetized with sodium amytal (0.2 ml. of a 1.8% solution). The livers are removed and placed into 4 ml. of hot 30% KOH, in a 15-ml. graduated centrifuge tube. After digestion, the glycogen is precipitated by the addition of 1.2 volumes of 95% ethanol. The tubes are heated until the mixture just begins to boil, cooled in an
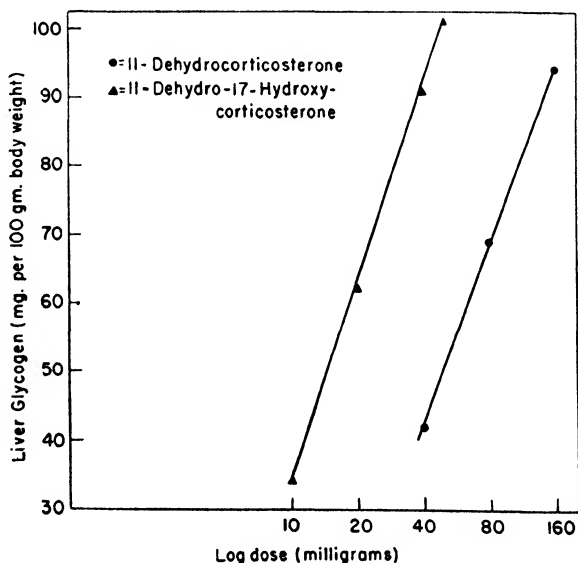
FIG. 8. Liver glycogen response of adrenalectomized mice to adrenal cortical steroids (Venning et al., 1946).

ice bath, and centrifuged. The supernatant liquid is poured off, and the tubes are allowed to drain. The sides of the tubes are washed with 0.5 ml. of ethanol and again allowed to drain. Final traces of ethanol are removed by heating the tubes in a hot-water bath for a few min. After hydrolysis of the glycogen, the glucose is determined by the method of Good *et al.* (1933). The glycogen is expressed as milligrams of liver glucose per 100 g. of body weight.

Table XXXI and Fig. 8 illustrate the influence of 11-dehydro-corticosterone and 11-dehydro-17-hydroxycorticosterone on the deposition of liver glycogen. The relationship is linear when the response is plotted against the logarithm of the dose. The method is sensitive enough to detect about 10 μg. of 11-dehydro-17-hydroxycorticosterone and about 40 μg. of 11-dehydrocorticosterone. Further, the indices of precision for the assay of these two compounds was calculated and found to be 0.137 and 0.222 respectively.

B. METHOD C OF EGGLESTON *et al.* (1946)

Male mice (18–24 g.) are bilaterally adrenalectomized under sodium pentobarbital anesthesia. The animals receive 0.25 ml. of Upjohn's

TABLE XXXII

The Liver Glycogen Responses of Adrenalectomized Mice to 11-Dehydro-17-hydroxy-corticosterone Using Methods C and D of Eggleston et al. (1946)

	Method D	Method C
	Fermentable sugar	Glycogen
Amount	mg./10 g. B.W.	mg./10 g. B.W.
μg.	±S.E.	±S.E.
0	2.8	0.7 ± 0.6
0.5		1.6 ± 0.03
1.0		2.0 ± 0.08
2.5		5.0 ± 0.7
5.0	6.4 ± 0.4	6.0 ± 0.6
10.0	11.0 ± 0.2	9.3 ± 1.0
12.5		11.1 ± 1.5
20.0	19.2 ± 0.8	12.0 ± 1.0
25.0		13.3 ± 0.7
40.0	24.8 ± 1.0	

aqueous adrenal cortical extract and are placed in a constant temperature room at 78°F. The animals are placed on the McCollum diet (see p. 351) and normal saline drinking water. At 4:30 P.M. on the fourth day after operation, the animals receive 0.4 ml. of Upjohn's aqueous adrenal cortical extract. At 8:30 A.M. the following morning hourly injection of the test material is begun. At each subcutaneous injection the animals receive 0.05 ml. of oil solution. Seven hourly injections are

made.   One-half hour after the last injection, the animals are weighed
and killed by snapping their necks.   The livers are quickly removed
and dropped into 5.0 ml. of hot 30% KOH, in a graduated 50-ml. cen-
trifuge tube.   The liver glycogen is determined by the method of Good
et al. (1933) using the sugar reagent of Shaffer-Hartman and Somogyi #2
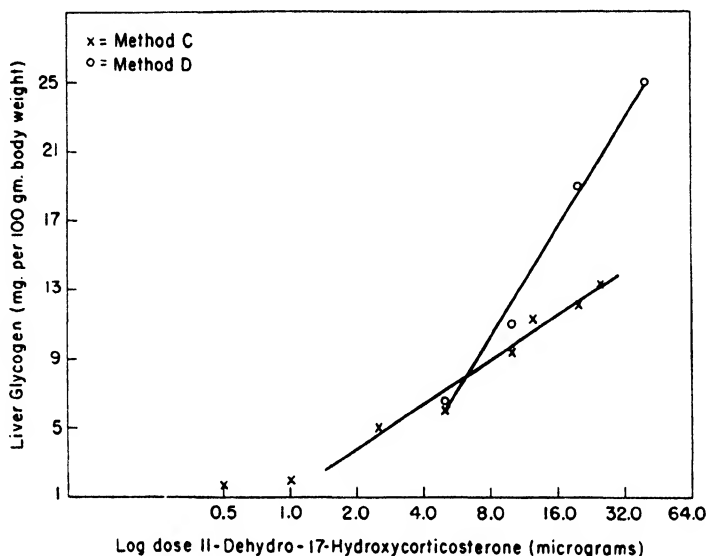
Fig. 9.   Liver glycogen response of adrenalectomized mice to 11-dehydro-17-hydroxy-
corticosterone (Eggleston *et al.*, 1946).

(Peters and VanSlyke, 1932).   The results are expressed as milligrams of
glucose per 10 g. of body weight (Table XXXII and Fig. 9).

C. METHOD D OF EGGLESTON *et al.* (1946)

The animals are killed as in method C.   The livers are removed
and dropped into a graduated Pyrex centrifuge tube containing 10 ml.
of N H_2SO_4.   After digesting the liver and cooling 0.8 ml. of 40% NaOH
and sufficient N NaOH is added to bring the solution to the first pink
color with phenolphthalein.   The proteins are precipitated by the method
of Somogyi (1930) and sugar is analyzed as described under method C.
Five milliliters of the supernatant are transferred to a graduated 15-ml.
centrifuge tube containing 5 ml. of washed and centrifuged Fleischmann's
baker's yeast.   The suspension is thoroughly mixed and allowed to
stand at room temperature for 1 hr.   The tube is centrifuged and 2 ml. of
the supernatant fluid is taken for the determination.   The total ferment-
able sugar is calculated by subtracting the nonfermentable reducing

substances from the total reducing substances. The results are expressed as total fermentable sugar per 10 g. of body weight.

Method D, which is less sensitive than C, has a precision about 10 times that of C.

Table XXXII and Fig. 9 illustrate the influence of 11-dehydro-17-hydroxycorticosterone on the deposition of liver glycogen. Method D is sensitive to 5 μg. and has a precision of $\lambda = 0.114$.

D. SUMMARY OF MOUSE GLYCOGEN METHODS

The use of the adrenalectomized mouse for adrenal cortical hormone carbohydrate activity is summarized in Table XXXIII. In the main

TABLE XXXIII

Adrenalectomized Mouse Glycogen Methods for the Assay of Adrenal Cortical Hormones

Compound	Slope b	Index of precision λ	Sensitivity μg.	Reference
11-Dehydro-17-hydroxycorticosterone (Method D)	21.04	0.114	5	Eggleston *et al.* (1946)
11-Dehydro-17-hydroxycorticosterone	95.9	0.137	10	Venning *et al.* (1946)
11-Dehydrocorticosterone	86.1	0.222	40	
11-Dehydro-17-hydroxycorticosterone	22.23	0.158	10	Dorfman *et al.* (1946)

these methods compare favorably as to precision with the rat methods presented previously (see p. 341). The mouse methods have a considerable advantage as to sensitivity, the order being about 10 to 20 times.

3. Muscle Work Test

The muscle work test for the assay of adrenal cortical steroids has been intensively studied by Ingle (1944b). The method is based upon the fact that muscular responsiveness is lost within a few hours following removal of the adrenal glands and that the work ability of the muscle can be maintained by the administration of suitable adrenal cortical extracts or pure compounds. Since the 11-oxygenated steroids are quite active and compounds such as desoxycorticosterone are practically without activity, it appears that the principal effect is on carbohydrate metabolism. The test is therefore classified in this section together with the glycogen methods.

A. METHOD OF INGLE (1944b)

For details and apparatus the reader is referred to original description (Ingle, 1944b). An abstract of the significant points in the method follows. Male rats, 180 ± 2 g. in weight are adrenalectomized and nephrectomized. The animal is fixed to a board and the gastrocnemius muscle stimulated weakly at first, and then the stimulus is gradually increased until a standard intensity is reached known to be optimal for sustaining the contraction of the muscle. Subcutaneous injection of the hormones in oil (0.5 ml. per dose) are done at the beginning, and again 6 hr. later. Adrenal cortical extracts are injected in aqueous ethanol solution (1.0 ml. per dose). Stimulation is continued until the muscle ceases to contract or for a period of 24 hr. The total number of contractions is recorded by means of a mechanical counter and is used as the index of efficiency.

B. RESULTS AND CONCLUSIONS

Table XXXIV presents a typical set of data on the muscle work test using the adrenalectomized-nephrectomized rat and the hormone 17-hydroxy-11-dehydrocorticosterone. Over the dosage range 0.16 to

TABLE XXXIV

The Influence of 17-Hydroxy-11-dehydrocorticosterone on the Muscle Work of the Adrenalectomized-Nephrectomized Rat (Ingle, 1944a)

Total dose mg.	Number of rats	Muscle work number of revolutions $\pm$S.E.
0	25	4000
0.16	45	9439 ± 415
0.20	45	11296 ± 342
0.25	45	12982 ± 382

TABLE XXXV

The Relative Activity of Adrenal Cortical Compounds on a Muscle Work Test (Data of Ingle and Kuizenga, 1945)

Compound	Relative activity in %
17-Hydroxy-11-dehydrocorticosterone	100
17-Hydroxycorticosterone	160
11-Dehydrocorticosterone	32
Corticosterone	46

0.25 mg. of hormone, the relationship is linear when the logarithm of the dose and response is considered (Fig. 10). The slope over this range is 18,250, and λ is 0.140.

By this test Ingle and Kuizenga (1945) compared the relative activity of 4 different adrenal cortical steroids. If 17-hydroxy-11-dehydrocorticosterone is considered the standard (100%) as in Table XXXV, 17-hydroxycorticosterone has a relative potency of 160%, 11-dehydrocorticosterone a value of 32%, and corticosterone 46%.

Log dose II-Dehydro-I7-Hydroxycorticosterone (milligrams)
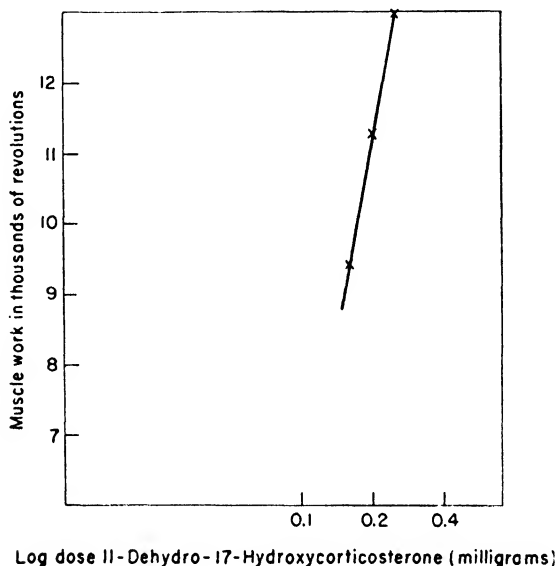
FIG. 10. Muscle work response of adrenalectomized-nephrectomized rats to 11-dehydro-17-hydroxycorticosterone (Ingle, 1944).

4. Anti-Insulin Action

This method described by Jensen and Grattan (1940) has not been studied in sufficient detail to judge its value. Attempts to adapt the method for quantitative work in the author's laboratory indicated that at least under the conditions used the method was too variable for precise assays.

VI. STRESS TESTS

These tests include the cold test, Everse-deFremery test, the swim test, the typhoid toxin test, and the animal restraint test. The cold test has been studied in the greatest detail.

1. Cold Tests

Hartman *et al.* (1931) demonstrated a significant difference in the sensitivity of normal and adrenalectomized rats exposed to low environmental temperatures and showed that the resistance of adrenalectomized

rats can be increased by the administration of adrenal cortical extracts. This phenomenon was adapted to the assay of adrenal cortical steroids by Selye and Schenker (1938), Venning *et al.* (1944), and Vogt (1943). Dorfman *et al.* (1946) have made an extensive study of the cold test as a means of assaying adrenal cortical hormones. The adrenalectomized mouse was not found to be satisfactory. Reasonable accuracy was found by using the operated rat.

A. METHOD OF DORFMAN *et al.* (1946)

Albino rats, 22 to 24 days of age and weighing between 35 and 52 g. were bilaterally adrenalectomized in one stage under ether anesthesia. The experiments were run between 12 and 24 hr. after operation. At this time the animals received the hormone either by injection or by stomach tube and were immediately placed in a wide mouth fruit jar. (1 pint size) containing a sheet of filter paper for bedding. The animals were transferred to a cold room kept at 5°C. and were observed at half-hour intervals until all had died.

Death was considered to have occurred in these studies when the animals had no visible respiration, showed low body temperature, and on stimulation showed no responsive movements.

It is imperative to run the unknown and standard simultaneously. In some runs the response decreases enormously. This happens about 1 out of every 3 trials, and seems to be independent of weight of animals, previous stress, or any known factor. This of course reduces the value of the test enormously.

B. RESULTS AND CONCLUSIONS

Table XXXVI and Fig. 11 illustrate the response of adrenalectomized rats exposed to low temperature when treated with two different samples

TABLE XXXVI

The Cold-Protecting Response of 11-Dehydrocorticosterone Acetate (Synthetic and Isolated) in Adrenalectomized Rats[a] (Dorfman, 1949c)

Dosage		Number of	
Synthetic	Isolated	adrenalectomized	Survival
μg.	μg.	rats	hours ± S.E.
0	0	10	9.7 ± 0.68
40	0	10	10.7 ± 0.88
80	0	10	11.9 ± 0.97
160	0	10	13.5 ± 1.17
320	0	10	15.4 ± 0.74
0	40	10	9.8 ± 0.81
0	80	10	11.3 ± 1.23
0	160	11	11.9 ± 0.90
0	320	11	14.2 ± 0.92

[a] Temperature 47° ± 1°F.

of 11-dehydrocorticosterone acetate. This represents a run under favorable conditions. Table XXXVII shows an example of the use of the cold test method employing the experimental design and calculations of Bliss (1944a, 1944b). In each case the unknown and standard
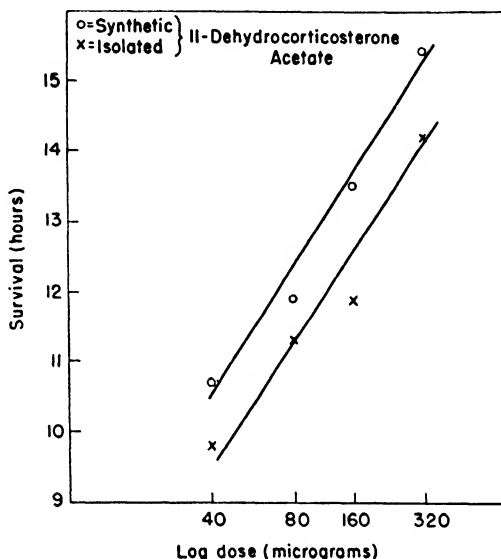
FIG. 11. Cold test using adrenalectomized rats (Dorfman, 1949c).

were significantly different as to slopes, and the index of precision, λ, varied from 0.276 to 0.410. The mean slope for the 4 trials was 11.5 $\pm$ 1.5, and the mean value of λ was 0.299 $\pm$ 0.072.

TABLE XXXVII

The Assay of 11-Dehydrocorticosterone Acetate Using a Cold Protection Test

$$\frac{\text{High dose of synthetic sample}}{\text{Low dose of synthetic sample}} = \frac{\text{High dose of isolated sample}}{\text{Low dose of isolated sample}}$$

$$N = 10$$

Dosage level µg.	b	λ	t	Potency ratio % $\pm$ S.E.
40; 80	10.8	0.379	0.276	74 $\pm$ 22
160; 320	15.5	0.187	0.410	72 $\pm$ 11
40; 160	8.1	0.337	0.333	50 $\pm$ 14
80; 320	11.4	0.291	0.119	76 $\pm$ 16

Table XXXVIII lists the relative activities of various adrenal cortical steroids in the cold test. When the potency of 11-dehydro-17-hydroxycorticosterone is set at 100%, 11-dehydrocorticosterone has a

relative potency of 33%, corticosterone, 9%, and 11-desoxycorticosterone acetate, 8%.

TABLE XXXVIII

Relative Potencies of Adrenal Cortical Steroids on the Basis of a Cold Test Expressed as the Percentage of the Activity of 11-Dehydro-17-hydroxycorticosterone

Compound	Relative potency %
11-Dehydro-17-hydroxycorticosterone	100
11-Dehydrocorticosterone	33
Corticosterone	9
11-Desoxycorticosterone acetate	8

Tables XXXIX–XLI summarize the various assay methods for adrenal cortical steroids. They indicate the material used, the test animal, the response, the index of precision (λ), and the sensitivity.

VII. SUMMARY

TABLE XXXIX

Adrenalectomized Rat Glycogen Methods for Assay of Adrenal Cortical Hormones

Material	Animal	Response	Slope $b \pm$ S.E.	Index of precision $\lambda \pm$ S.E.	Sensi- tivity µg.	References
11-Dehydro- cortico- sterone	Adrenal- ectomized rat	Liver glycogen	239 ± 25	0.095 ± 0.002	350	Pabst *et al.* (1947)
11-Dehydro- 17-Hydroxy- cortico- sterone	Adrenal- ectomized rat	Liver glycogen	213 ± 51	0.149 ± 0.031	350	Pabst *et al.* (1947)
Cortico- sterone	Adrenal- ectomized rat	Liver glycogen	198 ± 23	0.146 ± 0.024	125	Pabst *et al.* (1947)
17-Hydroxy- cortico- sterone	Adrenal- ectomized rat	Liver glycogen	203 ± 12	0.140 ± 0.014	125	Pabst *et al.* (1947)
Cortico- sterone	Adrenal- ectomized rat	Liver glycogen	2.06	0.112	300	Olson *et al.* (1944b)
11-Dehydro- cortico- sterone	Adrenal- ectomized rat	Liver glycogen	2.14	0.126	300	Olson *et al.* (1944b)
17-Hydroxy- cortico- sterone	Adrenal- ectomized rat	Liver glycogen	3.29	0.077	300	Olson *et al.* (1944b)
Adrenal cortical extract	Adrenal- ectomized rat	Liver glycogen	59.6	0.168	...	Reinecke and Kendall (1942)

TABLE XL

Adrenalectomized Mouse Glycogen Methods for Assay of Adrenalectomized Cortical Hormones

Material	Animal	Response	Slope b	Index of precision λ	Sensitivity μg.	References
11-Dehydro-17-Hydroxy-corticosterone	Adrenalectomized mouse (Method D)	Liver glycogen (fermentable sugar)	21.04	0.114	5	Eggleston *et al.* (1946)
11-Dehydro-17-Hydroxy-corticosterone	Adrenalectomized mouse	Liver glycogen	95.9	0.137	10	Venning *et al.* (1946)
11-Dehydro-corticosterone	Adrenalectomized mouse	Liver glycogen	86.1	0.222	40	Venning *et al.* (1946)
11-Dehydro-17-Hydroxy-corticosterone	Adrenalectomized mouse	Liver glycogen	22.23	0.158	10	Dorfman *et al.* (1946)

TABLE XLI

Miscellaneous Assay Methods for Adrenal Cortical Hormones

Material	Animal	Response	Slope b ± S.E.	Index of precision λ ± S.E.	Sensitivity μg.	References
Adrenal extract	Adrenalectomized drake	Survival	17.48	0.215 ± 0.036	...	Bülbring (1937)
11-Dehydro-corticosterone acetate	Adrenalectomized rat	Resistance to low temperatures	11.5 ± 1.5	0.299 ± 0.072	50	Dorfman *et al.* (1946)
Desoxy-corticosterone acetate	Adrenalectomized rat	Growth (6-day test)	33.16	0.154	360	Olson *et al.* (1944a)
11-Dehydro-17-Hydroxy-corticosterone	Adrenalectomized nephrectomized rat	Muscle work	18250	0.140	100	Ingle (1944b)

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The Chemical Assay of Steroids of the Androgen and Adrenocortical Hormone Groups

By R. K. CALLOW

CONTENTS

I. INTRODUCTION—CHEMICAL STRUCTURE OF THE ANDROGEN AND ADRENOCORTICAL HORMONE GROUPS IN RELATION TO ASSAY METHODS

Chemical assay methods applied to this group of compounds depend, both for separation and for final determination, on the presence of

certain characteristic structures and groups of atoms within the molecule. The compounds are complex, and in order that the ensuing discussion may be followed more easily it is as well to begin with a brief account of the structures which have to be considered and of the nomenclature employed.

The steroids have a tetracyclic carbon skeleton of 17 atoms. The androgens have two methyl groups attached to C-10 and C-13, and the corticosteroids have, in addition, a two-carbon atom chain attached to C-17. The system of numbering is shown in formula Ia.

The points of attachment of substituent groups important for our purposes are C-3, C-11, C-17, C-20, and C-21, which may carry hydroxyl groups (indicated in the name by x-hydroxy- or -x-ol) or ketonic oxygen

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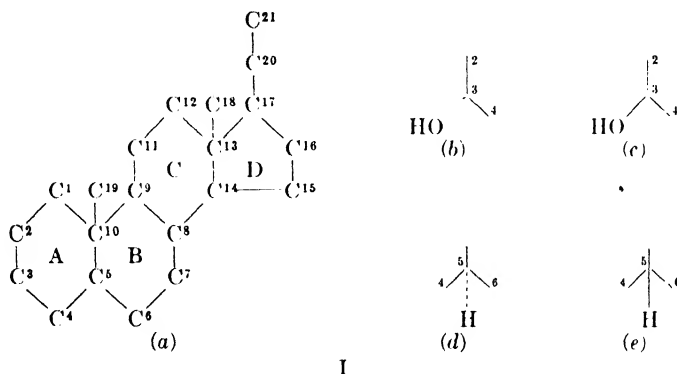
(x-keto- or -x-one). Double linkages between C-4 and C-5 (denoted by Δ^4-) or between C-5 and C-6 (Δ^5-) occur in some compounds of the series.

Certain stereoisomeric factors are important. As the four valencies of carbon are not coplanar, it will be clear that if C-3 carries the groups H and OH there are two possible isomers, one of which may be imagined to have the hydroxyl group below the plane of the rings (and of the paper) whilst the other has it projecting above. These are the 3(α)- and 3(β)-hydroxysteroids, represented with broken-line or full-line bonds, respectively, as in formulas Ib and Ic. The other important possibility of stereoisomerism is that due to the way in which rings A and B are linked at C-5. If the hydrogen atom on C-5 is below the plane of the paper the ring system is that of allopregnane or androstane (formula Id) or, if above it, the ring system is that of pregnane or etiocholane (formula Ie). Conventionally, structural formulas are written in a simplified line form.

The androgens are C_{19} compounds: the most active is testosterone

(systematic name, Δ^4-androsten-17-ol-3-one; formula II), but chemical assay methods are concerned principally with 17-ketosteroids, which are excreted metabolites, such as androsterone (androstan-3(α)-ol-17-one; formula III), isoandrosterone (androstan-3(β)-ol-17-one; formula IV), dehydroisoandrosterone (Δ^5-androstan-3(β)-ol-17-one; formula V) or etiocholan-3(α)-ol-17-one (formula VI), and with differentiation between the 3(α)- and 3(β)-isomers, exemplified by the compounds of formulas III and VI which are of the α type and IV and V which are of the β type.
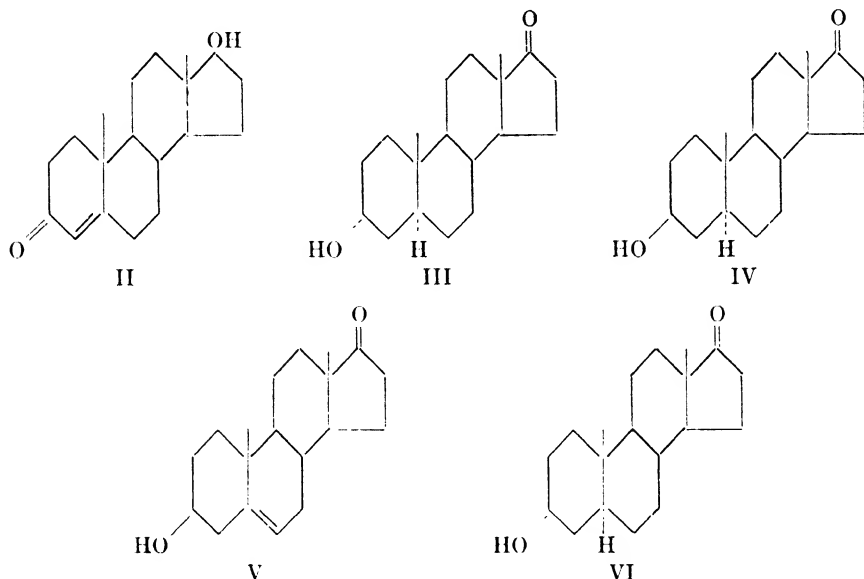
The corticosteroids are C_{21} compounds with, typically, oxygen on C-20 and C-21 and, commonly, on C-11. Compounds of importance are corticosterone (Δ^4-pregnene-11,21-diol-3,20-dione; formula VII), dehydrocorticosterone (Δ^4-pregnene-21-ol-3,11,20-trione; formula VIII), desoxycorticosterone (Δ^4-pregnene-21-ol-3,20-dione; formula IX) and 17-hydroxycorticosterone (Δ^4-pregnene-11,17,21-triol-3,20-dione; formula X). From the analytical chemical point of view the important characteristic features are the presence of oxygen on C-11 which is peculiar to corticosteroids, and the α-ketol or α-glycol groups attached to C-17 which, by reason of their sensitivity to oxidizing agents, provide a basis for quantitative estimation.

The assay of mixtures of these complex substances in biological material raises problems of extraction, of specificity of the reactions used for estimation, and of the type of compound, or individual compound, the amount of which it is important to measure. In the following sec-

tions these matters are discussed under the separate headings of androgens and of corticosteroids. In the case of androgens the development of the subject allows details to be given of recognized standard methods, with some appreciation of their value and significance. In the case of corticosteroids knowledge is still so incomplete that it would not be useful to give experimental details of methods which must be regarded as still subject to modification in the light of new work.

VII   VIII

IX   X
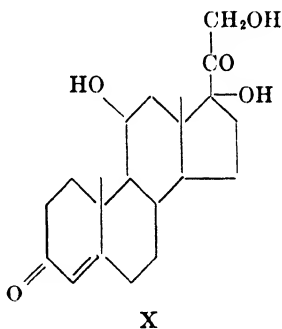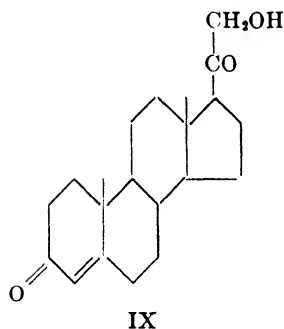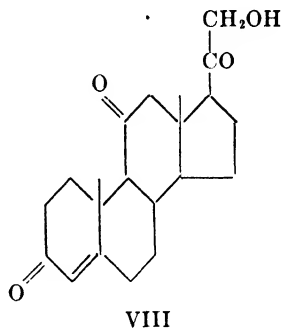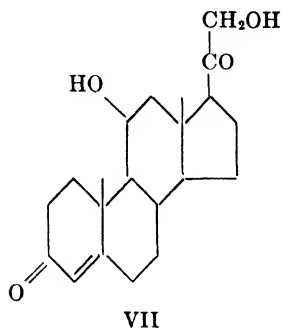
A technic applicable to both groups is that of infrared spectrometry, which is a physical rather than a chemical method. Nevertheless, developments in the last two years are so interesting and potentially important for the development of analytical schemes that a brief mention of them may be given here.

For an introduction to the subject reference may be made to a review by Jones (1948). Dobriner, Lieberman, Rhoads, Jones, Williams and Barnes (1948) described the use of infrared spectrometry as an analytical tool for the detection and characterization of small quantities of steroid metabolites in fractions of urinary extracts obtained in an extensive investigation by members of the same research group. They emphasize that it is highly probable that every organic compound has a unique infrared spectrum. Small, non-crystalline samples suffice for measure-

ment, and it is possible on the one hand to recognize known compounds in mixtures which are not too complex and to detect and follow components emerging from chromatographic adsorption columns.   Measurements in the region 1180 to 875 cm.$^{-1}$ are generally sufficient, for in this region marked individual differences occur.   On the other hand, it is also possible to draw conclusions as to the structure of unknown steroids from their absorption spectra.   Jones *et al.* (1949) have recently described the characterization of 11- and 12-oxygenated steroids, a matter of potential importance in the adrenocortical hormone group.

II. The Androgen Group

1. The Basis of Methods of Assay for Clinical Diagnostic Purposes

Chemical methods have a field of applicability which is restricted in practice almost completely to the examination of urine.   In considering the evolution of these methods it is important to give some attention to the rational bases underlying the methods proposed and their change as understanding of the biochemical problems has increased.

The chemical assay of androgens began when Zimmermann (1935, 1936) applied to 17-ketosteroids the color reaction given by methyleneketones, i.e., compounds containing the group ·CH$_2$·CO·, with *m*-dinitrobenzene and alkali.   He used first the pure compounds and then urine extracts, known to contain androsterone and dehydroisoandrosterone.   Zimmermann noted that the sum of the ketosteroids in urine exceeded the amount to be expected from biological assay.   When Callow *et al.* (1938) elaborated the Zimmermann reaction, they discussed at length the correlation of chemical and biological assays of urine extracts; they were concerned to show that chemical assay was at least as good as biological assay.   In fact, a significant degree of correlation was found between the colorimetric assay expressed in chromogenic equivalents of androsterone per liter of urine and the biological comb growth assay expressed in units of androgenic activity per liter.   A further justification for replacing the bioassay by chemical assay was that androgenic activity was associated with the ketonic fraction separated from urine extracts by Girard's reagent.

To assume diagnostic value for either 17-ketosteroid assays or biological androgen assays on urine involves in each case the assumption that the products excreted in the urine reflect the concentration of active primary materials in the organism.   In the case of bioassay, approximate correlation may be expected owing to the fact that androgenic activity is not specific to a very limited number of compounds which may be the circulating hormones, but extends to related compounds which are

evidently metabolites. In the case of chemical assay expectation of correlation is based on the observation that the end products of androgen metabolism are generally 17-ketosteroids. In either case, however, the assay is of a complex mixture, and only a dim and distorted picture of conditions in the body is obtained by these unspecific methods.

The matter is further complicated by the interrelation of the gonadal and adrenocortical hormones, for the adrenal cortex secretes androgenic material even in normal conditions, and does so particularly actively in pathological conditions, and the secretions give rise to androgenic and 17-ketonic metabolites—a conclusion which was reached at an early date (cf. discussion by Parkes, 1937) and confirmed by the isolation of 17-ketosteroids from the urine of ovariectomized women and of eunuchs (Hirschmann, 1939; Callow and Callow, 1940). For a fuller review of the subject reference may be made to the discussions by Dorfman (1948) of the metabolism of androgens, by Mason (1948) of urinary steroids in adrenal disease and the metabolism of adrenal hormones, and by Engstrom (1948) of the nature and significance of neutral steroids in human urine.

The full complexity of the steroid mixture in urine has recently become apparent as a result of the detailed and laborious investigation by Lieberman, Dobriner, and their colleagues (Dobriner, Lieberman and Rhoads, 1948; Lieberman *et al.*, 1948; Dobriner, Lieberman, Rhoads, Jones, Williams and Barnes, 1948; cf. the review by Lieberman and Dobriner, 1948). In the second paper of this series are described the chemical characteristics of forty-two ketosteroids from human urine. Reference must be made to the original paper for the detailed discussion of the relation of these urinary metabolites to their precursors, but in summary it may be said that half of the twenty-six identified compounds are "structurally labeled," retaining specific structural groups characteristic of the adrenocortical or corpus luteum hormones.

Further, it is a familiar fact that urine must first be hydrolyzed by boiling with acid before any considerable quantity of androgens or related steroids can be extracted. It has also been realized that this process leads to some decomposition and that certain compounds isolated, for instance chloroandrosterone, are probably artefacts. The only practical procedure has been to compromise by selecting conditions of hydrolysis which give maximum yields; nevertheless, some loss is unavoidable. Lieberman and Dobriner (1948) have recently reported on investigations of the conjugates of urinary steroids. Some are hydrolyzed at pH 1 in the cold, some are extractable by ether and hydrolyzed only on boiling, but the remaining fraction extractable only after the urine has been boiled with acid is relatively large, and, moreover, this third fraction includes all the steroids found in other fractions. There

is, therefore, full justification for the usual process of hydrolysis, despite the undetermined loss which occurs.

Finally, the large range of variation of the daily excretion of total 17-ketosteroids by different individuals of either sex and the impossibility of distinguishing the sex of a subject by 17-ketosteroid assay are notorious.

The various facts outlined above lead to the conclusion that assay of total 17-ketosteroids has only a preliminary diagnostic value.   Major deviations from the normal alone have diagnostic importance.   Therefore, particular interest attaches to efforts to discover more specific forms of assay, either by application of the reaction for 17-ketosteroids after fractionation of the urinary extracts, or by application of other reactions specific to particular types of compound which appear to be diagnostically significant, or by actual qualitative and quantitative analyses in greater detail of urine extracts to determine the individual compounds present and their amounts.

In the present-day stage of investigation of urinary steroids argument as to the significance of the nature or amount of a particular steroid occurring in urine is based, not on theoretical considerations derived from knowledge of the metabolic paths of known hormones, but rather on empirical correlation of observations of abnormal excreted products with clinical and pathological observations.   Crooke and R. K. Callow (1939) first found that a high level of urinary 17-ketosteroids indicated the presence of adrenocortical tumors, thus agreeing with earlier observations of high androgenic activity in the urine (cf. Simpson *et al.*, 1936), and, further, that the excess was largely due to a preponderance of dehydroisoandrosterone.

Later N. H. Callow and Crooke (1944) were able to quote confirmatory evidence, from other sources, of the association of high 17-ketosteroid excretion with adrenocortical tumors and to conclude that "the isolation of dehydroisoandrosterone from urine or the finding of a high ratio of β- to α-ketosteroids in urine is of fundamental importance in the diagnosis of adrenal tumors."

The first stage in chemical differentiation of 17-ketosteroids depends on the precipitability of steroids with a 3(β)-hydroxyl group, e.g., dehydroisoandrosterone, by digitonin, whereas the 3(α)-hydroxy compounds, e.g., androsterone, are not precipitated.   Methods of separation of α- and β-hydroxyketosteroids have been proposed, but they are not free from difficulty, and for purposes of rapid diagnosis with small amounts of urine a qualitative color test for dehydroisoandrosterone devised by Patterson (1947) has been successfully used for diagnosis of adrenocortical carcinoma and its differentiation from hyperplasia

(Broster and Patterson, 1948). Nielsen (1948) has attempted to develop the same reaction as a quantitative method.

A possible alternative method of differentiation of the steroids in urine extracts is provided by the method of Pincus (1943) for colorimetric determination, depending on the reaction of antimony trichloride with 3-hydroxysteroids, whether α or β, saturated in ring A. Dehydro-isoandrosterone does not react, and Salter et al. (1946) have suggested that separate determinations on the same urine of the total 17-keto-steroids by the Zimmermann method, and of the 17-ketosteroids reacting with the Pincus reagent, may be of diagnostic value.

It must be emphasized, however, that these chemical determinations are still diagnostically not completely specific. Dehydroisoandrosterone is not invariably present in great excess in the urine of tumor cases and a number of other compounds have been found in the urine of tumor cases which are absent or present only in small quantity in normal subjects. An attempt to carry out the analytical separation described by Dobriner, Lieberman, and Rhoads (1948) as a routine is clearly impracticable. On the other hand, it is obviously valuable to determine deviations from the normal pattern of steroid excretion with respect to as many compounds as possible. Hirschmann (1943) has pointed out the frequent occurrence of individual peculiarities among the few cases of adrenogenital syndrome in which detailed examination has been made, and only the determination of more chemical data for correlation with pathological data can make differential diagnosis possible.

The method of chromatographic-colorimetric analysis of urinary extracts introduced by Dingemanse et al. (1946) gives the best hope of scaling down a detailed analysis so that it comes within the bounds of a routine clinical laboratory procedure. In this method a relatively crude extract is adsorbed on a column of alumina and eluted. Deter-mination of 17-ketosteroids in successive equal volumes of eluate gives, when the values are plotted, an elution pattern with a series of peaks corresponding to known steroids, and qualitative and quantitative deviations of this pattern from the normal show great promise of diag-nostic significance.

A fair summary of the present position of chemical assay for pre-liminary diagnostic purposes of the androgen group in urine is, therefore, that determination of total 17-ketosteroids will give rather unspecific information of major abnormalities, that somewhat more specific informa-tion is given by separate determination of 3(α)- and 3(β)-hydroxyketo-steroids or, possibly, by combination of assays by the Zimmermann and Pincus methods, and that the method of Dingemanse et al. approaches most nearly the ideal: the Zimmermann method of determining 17-keto-

steroids is indispensable both as a preliminary test and for following more detailed fractionations: the Patterson test for dehydroisoandrosterone is a valuable qualitative test.

2. Specific Methods

A. THE DETERMINATION OF TOTAL 17-KETOSTEROIDS IN URINE EXTRACTS

i. *General.*   Numerous communications during the last twelve years have dealt with modifications in detail of the process of extracting and determining 17-ketosteroids in urine extracts.   Much of the work directed to obtaining the utmost accuracy and the highest "recovery" of urinary steroids is work of supererogation.   A moderate degree of reproducibility is sufficient for many purposes, higher accuracy is required only in serial determinations, when significance is attached to periodic variation, and in the control of fractionation processes.   These considerations will excuse the absence of any attempt to evaluate critically all the numerous variants of technic.   Much of what follows is based on the methods of the author and his associates, with modifications established by use in other laboratories (see Callow *et al.*, 1938; Callow *et al.*, 1939; and also Holtorff and Koch, 1940; Talbot, Butler, MacLachlan, and Jones, 1940; Zimmermann, 1944a–f, 1946; Cahen and Salter, 1944; Drekter *et al.*, 1947; Hamburger and Rasch, 1948).

ii. *Collection and Preservation of Urine.*   Except for special purposes a 24-hr. specimen is necessary owing to the diurnal variation in excretion. If extraction cannot be carried out within a day it is advisable to put a preservative into the vessel used for bulking collections.   Toluene (1% of final volume) is satisfactory provided that it does not come into contact with rubber or "composition" stoppers.   A known volume of concentrated hydrochloric acid may also be used.   Brilliant green or phenolic preservatives are unsatisfactory.

iii. *Hydrolysis and Extraction.*   These processes may be performed separately or combined in one operation.   Callow *et al.* (1939) studied the conditions affecting the process and described general methods of both types applicable to large or moderate volumes of urine.   For small-scale methods suitable for clinical assay, reference may be made to Patterson (1947) and to Hamburger and Rasch (1948).   The latter authors re-examined the conditions of extraction, treated the results statistically, and described methods of simultaneous and of separate hydrolysis and extraction suitable for one-fiftieth of a day's output of urine.

The following simple method is suitable for routine clinical assay.   To a 100-ml. portion of the 24-hr. specimen of urine is added 15 ml. of concentrated hydrochloric acid, and the mixture is heated to boiling in an

all-glass reflux apparatus, boiled for 15 min., and then cooled by immersing the flask in water. The hydrolyzed urine is then extracted twice with 100 ml. portions of peroxide-free ether. The combined extracts are washed twice with 20 ml. of 2 N sodium hydroxide and three times with 10 ml. of water. The addition of a pinch of sodium dithionite ($Na_2S_2O_4$) to the last wash water is recommended by some workers. This procedure, based on that of Talbot, Wolfe, MacLachlan, Karush, and Butler (1940) is stated to facilitate the removal of substances of quinone type which are alkali-soluble when reduced, but not when in the oxidized form, and gives a less colored final extract. The washed extract is evaporated to dryness and the residue left overnight in a vacuum desiccator.

iv. *Colorimetry by the Zimmermann Reaction.* (*1*) *Reagents.* *Ethyl alcohol.* Some commercial grades of absolute alcohol are suitable. Usually, however, purification from aldehydes is necessary. The alcohol is treated with 4 g./l. of *m*-phenylenediamine hydrochloride, allowed to stand in the dark for a week, with occasional shaking, and then distilled, rejecting the head and tail fractions (Rowe and Phelps, 1924). Holtorff and Koch (1940) use redistilled 95% alcohol.

m-Dinitrobenzene. Purified material which gives a pink or red color with potassium hydroxide is further purified as follows. A batch of 20 g. is dissolved in 750 ml. of 95% alcohol warmed to 40°C. and 100 ml. of 2 N NaOH is added. After 5 min. the solution is cooled and 2.5 l. of water is added. The precipitate is collected in a Büchner funnel, washed very thoroughly with water, sucked dry, and recrystallized twice in succession from 120 ml. and 80 ml. of absolute alcohol. The material should be well crystallized in almost colorless needles, m.p. 90.5–91°C. A 1% alcoholic solution mixed with an equal volume of aqueous 2 N NaOH should give no color after standing for 1 hr. The reagent is a 2% (w/v) solution in absolute alcohol. Stored in a stoppered bottle in the dark it is stable for 10–14 days.

Alcoholic potassium hydroxide. Pure potassium hydroxide (9 g.) is stirred with 50 ml. of absolute alcohol, the solution filtered, and the concentration, which should be between 2.48 and 2.52 N, is checked by titration; it may be adjusted by dilution if necessary. The solution is stable for 2–5 days if stored in a refrigerator; it must be discarded as soon as the faintest color is perceptible.

Aqueous potassium hydroxide. This should be free from carbonate and between 2.98 and 3.02 N (Zimmermann, 1944b), or between 4.98 and 5.02 N (Holtorff and Koch, 1940). The solution may be stored for a month.

(*2*) *Reaction.* Test tubes should have been thoroughly cleaned, e.g., with nitric and chromic acid mixture. Into one tube, to serve as blank,

are measured out in succession, from 1-ml. pipets graduated to 0.01 ml., 0.2 ml. of alcohol, 0.2 ml. of *m*-dinitrobenzene solution, and 0.2 ml. of alcoholic potassium hydroxide solution.   Holtorff and Koch use 0.2 ml., and Zimmermann uses 0.1 ml. of aqueous potassium hydroxide.

Into a second tube is measured out 0.2 ml. of the alcoholic solution of the substance to be tested followed by the reagents.   The time of adding the potassium hydroxide is noted.   The tubes are stoppered, well shaken, and kept at $25 \pm 0.1°C$. in dull, diffused light.   After 1 hr., 10 ml. of alcohol is added to each tube, and the contents mixed and transferred to the cells of the colorimeter, which are closed by cover slips or other form of suitable lids.

(*3*) *Measurement.*   A type of colorimeter or absorptiometer is used which allows absolute measurements to be made with a green filter (max. transmission about 520 mμ) and a violet filter (max. transmission about 430 mμ).   Apparatus which has been used includes the Pulfrich stufen-photometer with Zeiss S53 and S43 filters, the Hilger Spekker absorptiometer with Ilford spectrum green or 604 and spectrum violet or 601 filters, the Evelyn photoelectric colorimeter with Rubicon Nos. 520 and 420 filters, and other instruments.   Callow *et al.* (1938) showed that it was possible to use plunger type colorimeters with 0.001 N permanganate as an artificial standard for comparison.

A calibration curve should be constructed for readings, using the green filter, with suitable weights (say 0.05, 0.1, and 0.15 mg.) of pure androsterone or dehydroisoandrosterone—these two substances give closely similar values—and checked occasionally when fresh batches of reagents are brought into use.

Measurements on urine extracts should be made with an amount which gives a colorimeter reading on the straight portion of the calibration curve, and it may be necessary to repeat the measurement with a more dilute solution of the urine extract.

(*4*) *Color correction.*   Measurements should be made also with the violet filter and a correction factor applied to the reading with the green filter if it is thought necessary.   This question of correction has been much discussed.   It is known that substances in urine extracts give, with the Zimmermann reagent, colors with general absorption, and it is obvious that the absorption at 520 mμ will be affected by such interfering substances and will no longer be proportional to the content of 17-keto-steroids.   While some extracts give clean colors, the 17-ketosteroid curve with its maximum in the green standing out boldly, others give colors with notable absorption in the violet, and numerical comparisons of the absorption values in the green are obviously misleading.   Callow *et al.* (1938) took the easy course of simply neglecting such results, where the

values of the ratio E_v/E_g exceeded 0.8. Urines giving colors of this type had low contents of 17-ketosteroids and were, from their point of view, at that time, of little interest.

If the nature of the investigation justifies an attempt to find the true 17-ketosteroid content from the colorimetry of a crude extract, a correction may be applied. It is also expedient to give "corrected" figures for routine clinical assays, instead of having to add an involved explanation to the clinician of why a figure is apparently high but not really significant of high 17-ketosteroid excretion. Fraser *et al.* (1941), Talbot *et al.* (1942), and Engstrom and Mason (1943) have applied such corrections, based on theoretical considerations discussed by Gibson and Evans (1937) (cf. Gibson and Evelyn, 1938). Engstrom and Mason found that, using the absolute alcohol technic, the use of a correction formula gave results for 17-ketosteroid content of crude extracts in close agreement with those obtained with the ketonic fractions separated from the same extracts. The aqueous alcohol technic (Holtorff and Koch, 1940) could not be used with a correction factor, since Beer's law was not followed with crude extracts.

The formula to be applied is:

$$\text{Corrected green reading} = \frac{K_i E_g - E_v}{K_i - K_s}$$

where E_g and E_v are the extinction coefficients of the test solution with green and violet filters, respectively, and K_i and K_s are the values of E_v/E_g for the interfering chromogens and for pure 17-ketosteroid, respectively. (Note the corrigendum by Talbot *et al.*, 1943.) The values of K_i and K_s determined on non-ketonic fractions of crude extracts and on androsterone (or dehydroisoandrosterone), respectively, will, of course, vary with the apparatus and technic employed and must be determined for any particular setup. Thus, Engstrom and Mason (1943) found an average value for K_i of 1.5 (range 1.3 to 1.8) and for K_s of 0.4. Hamburger and Rasch (1948) found $K_i = 1.27$; $K_s = 0.6$.

v. *Polarographic Determination.* Wolfe *et al.* (1940) investigated the applicability of the polarographic method of analysis to ketosteroids. As was to be expected, the method could only be used directly with α,β-unsaturated ketones, but satisfactory derivatives of saturated 17-ketosteroids were furnished by the reaction products with Girard's reagent T, (acethydrazidetrimethylammonium chloride) which are of the form: $>C:N\cdot NHCOCH_2N^+(CH_3)_3Cl^-$.

A number of improvements in technic were suggested by Barnett, Henly, and Morris (1946), the most important being to decrease the rate

of rise of applied voltage and to remove interfering material (probably 3- and 20-ketosteroids) from the extracts by oxidation with permanganate and iodate.

In subsequent papers, alternatives to the Girard reagent were investigated, without finding anything better (Barnett and Morris, 1946) and a detailed comparison was made of a series of determinations by the polarographic and the Callow-Zimmermann methods (Barnett, Henly, Morris, and Warren, 1946). The colorimetric method gave somewhat higher results than the polarographic method, and its standard deviation was also higher. With application of the color correction of Talbot, Berman, and MacLachlan (1942) to the colorimetric results, correspondence was much closer, but a few high, discrepant colorimetric values were obtained (Morris, 1948).

An interesting development of the polarographic method was investigated by Hershberg *et al.* (1941). Oxidation of dehydroisoandrosterone or other 3-hydroxy-Δ^5-steroids by aluminium *tert.* butoxide and acetone (Oppenauer reaction) yields the corresponding 3-keto-Δ^4-steroids, e.g., dehydroisoandrosterone yields Δ^4-androstene-3,17-dione. The condensation product of this α,β-unsaturated ketone with Girard's reagent gives rise to a characteristic cathodic wave at a half-wave potential of -1.25 volts, distinguishable from the value of -1.45 volts obtained with 17-ketosteroid derivatives. A micromethod for determination of the dehydroisoandrosterone content of a sample containing about 0.5 mg. of total 17-ketosteroid was elaborated on this basis.

A small number of laboratories using polarographic technics may continue to apply the methods which have been outlined above, but colorimetric methods are undoubtedly more favored; for this reason it does not seem justifiable to give detailed experimental directions here, and for these the original papers should be consulted.

B. FRACTIONATION OF URINE EXTRACTS IN CONJUNCTION WITH 17-KETO-STEROID ESTIMATIONS

In the case of routine clinical assays a determination of total 17-ketosteroids on an unfractionated extract will generally give all the information required. If, however, an abnormal value is obtained, or the clinical data required more extended investigation, fractionation is indicated, and for other special purposes it is essential. Thus, special interest may be attached to the true 17-ketosteroid content of an extract, and it may be more satisfactory to separate the ketonic fraction rather than to apply a color correction. Again, the special diagnostic significance of a high output of 3(β)-hydroxysteroids has been indicated, and a separation of α and β fractions will be valuable. Separation of the non-ketonic fraction

into alcoholic and non-alcoholic steroids and separation of the former into α and β fractions is a procedure only likely to be useful in large-scale investigations.

i. *Separation of a Ketonic Fraction.* Callow *et al.* (1938) used Girard's reagent T (trimethylacethydrazideammonium chloride) for this separation, and the method was developed by Talbot, Butler, and MacLachlan (1940) and Pincus and Pearlman (1941). The last-named authors take the crude extract from 100 ml. urine and dry it in a Pyrex test tube over calcium chloride in a vacuum desiccator; 0.5 ml. glacial acetic acid and about 100 mg. Girard's reagent T are added; the tube is loosely stoppered with a cork wrapped in aluminum foil and placed in an oil bath at 90–100°C. for 20 min. After cooling, 15 ml. of ice water is added, and the mixture is immediately transferred to a small separatory funnel. Sufficient 2.5 N NaOH is added to neutralize nine-tenths of the acetic acid ⋅ and three extractions with 20-ml. portions of ether are made. The ether extracts are combined and washed once with 10 ml. ice water. This wash is combined with the rest of the aqueous ketonic fraction. The ether is washed with 10 ml. 0.25 N sodium carbonate solution and with three 10-ml. portions of water. The ether contains the non-ketonic fraction. The aqueous ketonic fraction is acidified with 3 ml. concentrated hydrochloric acid, allowed to stand at room temperature for 2 hr., and then extracted three times with 20-ml. portions of ether. The ether is washed with 10 ml. 0.25 N sodium carbonate solution and three times with 10-ml. portions of water. The ether contains the ketonic fraction. Since the ketone condensation product hydrolyzes spontaneously if allowed to stand at room temperature, it is important to carry out the separation as rapidly as possible.

Dobriner, Lieberman, and Rhoads (1948) use, as part of a large-scale separation, a technic in which 10 ml. of absolute alcohol, 1.25 ml. of glacial acetic acid, and 1 g. of reagent T are employed for each 100 mg. of ketosteroid present. The ketones were recovered from the hydrazones by acid hydrolysis at pH 1 at room temperature for at least 16 hr., followed by continuous extraction of the hydrolysates with ether for 24 hr. The authors state that the hydrolysis of certain steroid hydrazones is not always complete in a period shorter than 16 hr. at pH 1, and the exhaustive extraction with ether is necessary to effect complete recovery of certain highly oxygenated water-soluble steroids. They also point out that the Girard separation is not strictly quantitative and that, if pure compounds are to be isolated at a later stage, repetition on both fractions may be an advantage.

ii. *Separation of α and β Ketosteroids [3(α)- and 3(β)-Hydroxyketosteroids].* Processes for the separation of α and β ketosteroids on a small

scale have been described by Baumann and Metzger (1940), Talbot, Butler, and Berman (1942), Frame (1944), Pincus (1945), and Butt *et al.* (1948). The procedure of the last-named authors is as follows.

An amount of urine extract containing 1.0–1.5 mg. of 17-ketosteroid is transferred to a graduated centrifuge tube and evaporated to dryness under reduced pressure. Warm digitonin solution (0.75 ml. of 1% solution in 90% (v/v) alcohol) is added and the mixture quickly heated to boiling in order to dissolve as much of the material as possible. The tube is stoppered and left in a refrigerator overnight. A total of 10 ml. of peroxide-free ether is then added in small portions, stirring between each addition, and allowing the precipitated digitonin and digitonides to flocculate before adding the last 2–3 ml. After centrifuging, the supernatant is decanted into a separatory funnel, and the precipitate is washed three times with 5-ml. portions of ether, stirring and centrifuging each time. The combined supernatants are washed three times with 5 ml. portions of water and then evaporated to dryness under reduced pressure. The residue constitutes the α fraction. The precipitate is dissolved in 0.25 ml. dry pyridine, warmed to 60–70°C. for 3 min., cooled, and 5 ml. of ether added in portions, stirring and allowing the precipitate to flocculate. The mixture is centrifuged, and the precipitate is again treated with pyridine and ether, and the final residue washed twice with 5-ml. portions of ether. The combined extracts are washed twice with 5-ml. portions of 2 N sulfuric acid, three times with 5-ml. portions of water and then evaporated to dryness under reduced pressure. The residue is the β fraction.

For larger scale separations, Dobriner, Lieberman, and Rhoads (1948) used the procedure of Butler and Marrian (1938) with minor modifications.

It may be mentioned that commercial samples of digitonin vary in quality and may be unsuitable for use in these fractionations. A preliminary trial with dehydroisoandrosterone is advisable before bringing a batch into use.

iii. *Fractionation of the Non-Ketonic Fraction—Division into Alcoholic and Non-Alcoholic Fractions.* As has been said above, this is a procedure usefully applicable only to larger scale separations. Pincus and Pearlman (1941) have described a small-scale procedure of esterification with succinic anhydride. Dobriner, Lieberman, and Rhoads (1948) have described more recently separation with the use of phthalic anhydride.

C. COLORIMETRIC DETERMINATION OF DEHYDROISOANDROSTERONE

Dirscherl and Zilliken (1943), in a paper not generally available owing to war conditions, described a color reaction of dehydroisoandrosterone

occurring when it was dissolved in sulfuric acid and water added.   Nielsen (1948) developed this reaction into a method of quantitative determination of the pure substance and Nielsen *et al.* (1948) applied the method to examination of urine from a case of adrenocortical adenoma.

The reaction with the pure substance is carried out by taking 0.4 ml. of 0.05% solution in absolute alcohol in a test tube.   The test tube is placed in ice water, and 2.0 ml. of sulfuric acid is added.   The solution is stirred and heated in a boiling water bath for 90 sec.   The tube is replaced in ice water, and, after diluting with 8.0 ml. of 25% or 37.5% (v/v) sulfuric acid, the extinction of the solution at 600 mμ is determined with a spectrophotometer.   With 25% acid the reading can be made immediately, with 37.5% acid after 40 min.   A determination with 0.2 mg. of dehydroisoandrosterone is carried out as control.

In applying this method to a urine extract an erroneously high figure was obtained, evidently as a result of color due to impurities, and the true content was probably about half that indicated.   A more promising result was obtained by separation of the urine extract into α and β fractions by the method of Frame (1944).   Examination of the β fraction showed that it contained 17-ketosteroids corresponding to 68%, and dehydroisoandrosterone to the extent of 65% of the total 17-ketosteroids.

The method of Nielsen can be regarded as promising, but by no means developed to the point of being a satisfactory routine method.   It would seem to be much better, if a urine extract shows a high 17-ketosteroid content, and evidence of the amount of dehydroisoandrosterone present is sought, to use the Dirscherl-Zilliken color reaction as a qualitative test in the form developed independently by Patterson (1947) and then to seek confirmation either by assay of 17-ketosteroids in α and β fractions, or, perhaps preferably, to undertake a more detailed analysis by the chromatographic-colorimetric method.   The Patterson reaction is described in detail below.

D. THE PATTERSON COLOR REACTION FOR DEHYDROISOANDROSTERONE

Patterson (1947) describes in detail the preferred methods of preservation, hydrolysis, and extraction of urine to be tested.   It can, however, be assumed that the neutral fraction of a urine extract suitably prepared for estimation of 17-ketosteroids may be used.   If, for instance, a 25-ml. portion of urine from a 24-hr. specimen is worked up to yield an alcoholic extract, one-fifth of this extract is taken in a dry test tube and evaporated to dryness in a vacuum desiccator.   To the residue is added 1 ml. of concentrated sulfuric acid, and the tube is shaken until the extract is completely dissolved.   The tube is then kept at 25°C. for 20 min., and 1 ml.

of distilled water is added drop by drop down the side while the tube is kept cool by shaking in cold water. The tube is then heated in a boiling water bath for 1 min. A blue or blue-violet color developing in the final stage indicates the presence of excess dehydroisoandrosterone or closely related steroids. The color is very stable and unaltered by several minutes' heating.

The reaction is also given by isoandrosterone and by a third substance, possibly $\Delta^{3,5}$-androstadien-17-one, present in extracts of tumor urine. The reaction is claimed to be sensitive to less than 0.1 mg. dehydroisoandrosterone and in a case of adrenal carcinoma with a 17-ketosteroid excretion of 1980 mg. a day the reaction was distinct with the equivalent of 0.1 ml. of urine (Broster and Patterson, 1948).

E. THE PINCUS REACTION

Pincus (1943) found that an intense blue color (with an absorption maximum at 610 mμ) was developed by certain steroids when heated with antimony trichloride, either as a concentrated solution or as an amorphous solid, and diluted with acetic acid. The reaction is given by androsterone and its isomers (isoandrosterone, etiocholan-3(α)-ol-17-one) and by androsten-17-one, but not by a variety of other steroid derivatives, in particular dehydroisoandrosterone.

The reagent is prepared by dissolving antimony trichloride in a 9:1 mixture of acetic acid and acetic anhydride in the proportion of 3.8 g. of solid to 1 ml. of solvent. To the dried steroid (0.1 mg., or the equivalent amount of urine extract) in a test tube, 0.2 ml. of the reagent is added drop by drop from a microburet. The tube is stoppered and heated in a boiling water bath for 20 min. The tube is thoroughly shaken within 1 min. in order to ensure complete solution. After heating, the tube is cooled, and the solution is diluted with glacial acetic acid, added slowly with shaking. The typical color develops and reaches a maximum in 40–60 min. at room temperature.

There are few records of the application of this reaction, promising though it may appear for the determination of 3-hydroxysteroids other than dehydroisoandrosterone. Salter *et al.* (1946) carried out a number of determinations by this method in parallel with determinations by a modified Zimmermann method and found, rather surprisingly, that values were generally the same. In certain tumor cases, however, there were marked differences, and they concluded that if the modified Zimmermann method gave a value of over 100 mg. per day and the Pincus method gave a total value of less than 80% of this, the circumstances were "highly suggestive of tumor."

F. THE MICROMETHOD OF DINGEMANSE AND CO-WORKERS FOR CHROMATO-GRAPHIC SEPARATION OF URINARY 17-KETOSTEROIDS

The use of this method has not been recorded by many workers since it was first published (Dingemanse *et al.*), but it evidently has considerable potential value as a routine method for clinical diagnostic purposes. It enables a qualitative or semiquantitative comparison to be made of from seven to nine components of the neutral fraction of urine extracts and is a tremendous advance on the unspecific methods so far described. Nielsen *et al.* (1948) had no great success with the method in examining extracts from the urine of a patient with a virilizing adenoma of the adrenal cortex. Nevertheless, Devis and Férin (1948) used the method successfully, and Robinson and Goulden (1949) have given details of technic which should assist other workers to reproduce the results obtained. They emphasized the necessity of standardizing the alumina used for chromatography and were able to obtain closely reproducible results. The following account is based largely on their paper.

Urine extracts are prepared by the usual method and a preliminary estimate of 17-ketosteroid content is made. A quantity of the alcoholic solution containing about 5 mg. (and constituting a known proportion of the 24-hr. excretion) is taken and evaporated to dryness. The last traces of alcohol are removed by two successive evaporations after addition of pure benzene and the residue is then dissolved in 50 ml. of dry, thiophene-free benzene.

It was found essential to choose an alumina of appropriate activity: Spence Type H alumina was suitable after exposure to the air in thin layers for up to 3 days. The adsorptive power was checked at intervals by the method of Brockmann and Schodder (1941), as follows. Sudan red (20 mg.) and Sudan yellow (20 mg.) both purified by recrystallization, are dissolved in 10 ml. of benzene, and the solution is made up to 50 ml. with light petroleum. For standardization, 1 ml. of this dye solution is adsorbed on a column 50x14 mm. prepared from a suspension of the alumina (7 g.) in benzene-light petroleum (1:4) and eluted with 50 ml. of the same mixed solvent. The alumina is considered to have suitable adsorptive power when the yellow band reaches the bottom of the column while the red band moves down about 10 mm.

The alumina columns for adsorption of the urine extracts are prepared in tubes of 14 mm. internal diameter fitted with taps, and a plug of cotton wool is inserted above the tap as support. The alumina (13.0 g.) is poured into the tube in benzene suspension, and packing takes place by gravity alone, giving a column about 10 cm. high with space for 60–70 ml. of solvent above. The benzene level is allowed to fall to the top of the alumina, and the solution of extract is then poured on. The benzene

eluate constitutes fraction 0. Elution is continued with (1) 350 ml. benzene, (2) 1.2 l. 0.1% (v/v) ethyl alcohol in benzene, (3) 500 ml. 0.5% (v/v) ethyl alcohol in benzene, and (4) 100 ml. ethyl alcohol. The tube
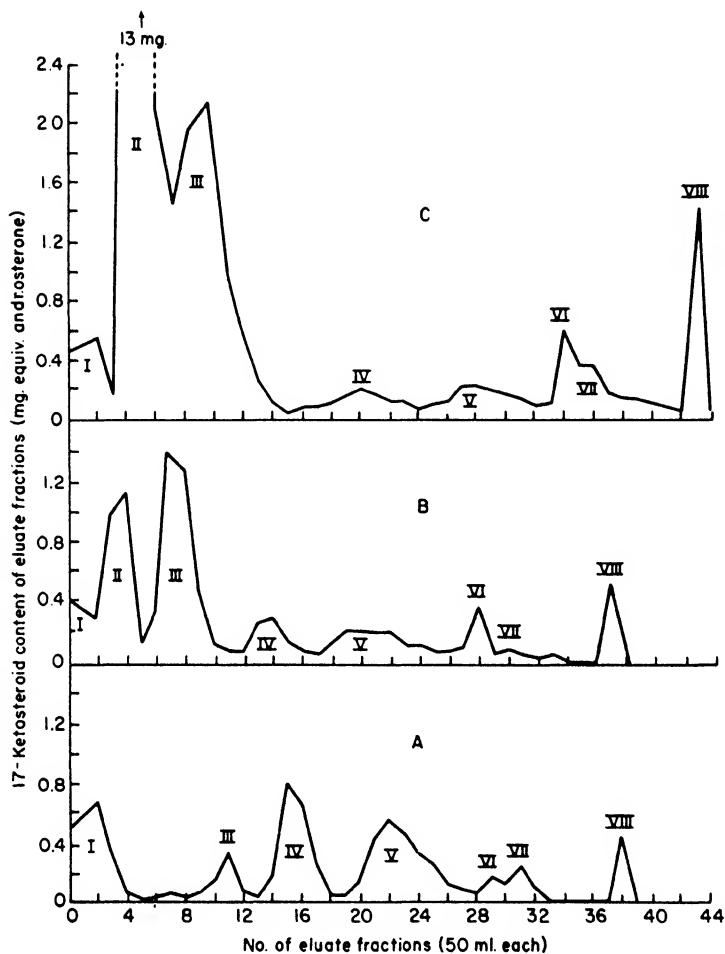
FIG. 1. Chromatographic-colorimetric assays by the method of Dingemanse *et al.* (1946). (Redrawn from Dingemanse *et al.* (1946).)

A. Extract of 500 ml. urine from a normal woman.

B. Extract of 100 ml. urine from a "female intersex Type A" suggestive of adrenal enlargement.

C. Extract of 120 ml. urine from a patient with a malignant adrenal tumor.

above the alumina is kept full of liquid except when changing eluents, and the eluate is collected in 50-ml. fractions numbered 1–43.

The fractions are evaporated to dryness, each taken up in 2 ml. of

alcohol, and the 17-ketosteroid content estimated by the m-dinitrobenzene method.   A graph is then constructed with abscissas corresponding to the serial number of the eluate fraction and 17-ketosteroid values plotted as ordinates.   The curve obtained by joining these points consists of a series of peaks, more or less prominent in different extracts.   Numbering these peaks I–VIII, constituents have been identified in five of them, as follows.   I, $\Delta^{3,5}$-androstadien-17-one, 3-chloro-Δ^5-androsten-17-one and Δ^2- or Δ^3-androsten-17-one; II, i-androsten-6-ol-17-one (Dingemanse *et al.* (1948); Barton and Klyne (1948)); II, dehydroisoandrosterone and iso-androsterone;   IV, androsterone;   V, etiocholan-3(α)-ol-17-one.   Three typical graphs obtained by Dingemanse *et al.* (1946) are reproduced in Fig. 1.   Robinson and Goulden observed an additional peak (IIa) in some cases.   The newly recognized compound in peak II was invariably encountered by Dingemanse *et al.* in the urines of patients with adrenal dysfunction (including malignant tumors) and virilism and intersexes with virilism.   Recognition of the peaks in case of difficulty is assisted by applying Patterson's test for dehydroisoandrosterone, which is positive with peak III.

G. PAPER PARTITION CHROMATOGRAPHY OF KETOSTEROIDS

Zaffaroni *et al.* (1949) have succeeded in applying the method of paper chromatography to the separation and recognition of ketosteroids in the form of their hydrazones with Girard's reagent T.   Ten of these compounds have been examined, including the four important urinary steroids —androsterone, isoandrosterone, dehydroisoandrosterone, and etiocholan-3(α)-ol-17-one.   Unfortunately, with the technic so far used, the number of carbonyl groups in the steroid is the overwhelming factor determining the R_F values, i.e., rate of travel of substance relative to the solvent front. Androsterone and isoandrosterone hydrazones with reagent T have R_F values of 0.42, whereas the dehydroisoandrosterone and etiocholan-3(α)-ol-17-one compounds have $R_F = 0.49$ in n-butanol saturated with water. Although the authors quite legitimately foresee useful application to the recognition of some of the components of urine extracts, it is clear that further elaboration of the method, for example by a preliminary fractionation into 3(α)- and 3(β)-hydroxysteroids, will be necessary if certain steroids, the relative amount of which is regarded as diagnostically significant, e.g., dehydroisoandrosterone, are to be estimated.   On the other hand, the method may be, as the authors suggest, a useful aid in determining the homogeneity of fractions obtained in the separation of ketosteroid mixtures by adsorption chromatography.   Again, the separation of urinary extracts into mono-, di-, and higher ketonic fractions is satisfactory within certain limitations and the promised study of the

pattern of ketosteroid excretion in a variety of endocrine disorders should be of great interest.

Camber (1949), whose work on the histochemical demonstration of ketosteroids in the adrenal cortex is mentioned below, has suggested the use of aryl hydrazones of ketosteroids, e.g., the 2-hydroxy-3-naphthoic acid hydrazones, for chromatographic purposes. Such compounds might be detected by fluorescence in ultraviolet light or by coupling with diazonium compounds.

H. MISCELLANEOUS MATERIALS

The subject of assay of 17-ketosteroids in human urine dominates the field, but the application of chemical assay to other purposes deserves a brief review. In lower animals the metabolism of androgens does not seem, in general, to resemble that in man, and application of methods suitable for human urine has met with difficulties. Again, for the study of hormone activity and metabolism in the body, determination of steroids of the androgen group in blood and tissues would be of great value, but the complexity of the material makes chemical assay methods extremely difficult.

i. *Ketosteroids in the Urine of Lower Animals.* Little or nothing comparable with the work on human urine has been carried out on the urine of lower animals. Butz and Hall (1938) and Marker (1939) investigated the urine of bulls and cows, and the latter isolated androsterone and dehydroandrosterone. Whitten (1943) investigated the urine of sheep, Green *et al.* (1942), and Green and Winters (1945) that of boars, and Kimeldorf (1948a, b) that of rabbits; in all cases variants of the Zimmermann reaction were used. Green *et al.* found 17-ketosteroid in amounts equivalent to 0.5–3.5 mg. androsterone per 24 hr. in the urine of boars, according to age and other conditions. After castration the values fell, but did not reach zero, although the androgenic activity fell below the limit of measurement. Kimeldorf found that it was necessary, to separate the ketonic fraction in order to eliminate interfering chromogens; when this had been done the color given was closely similar to that given by pure 17-ketosteroids. The average 17-ketosteroid output of rabbits was about 1 mg. per day; it was decreased by castration or lowered adrenocortical activity.

ii. *Ketosteroids in Blood and Tissue.* Zimmermann (1944g) described the application of his colorimetric reaction to blood, but admitted (Zimmermann, 1946) that insufficient experience had been obtained to judge of the value and dependability.

The method of extraction is as follows. Serum (10 ml.) is treated with sodium tungstate (5 ml.) and $\frac{2}{3}$ N sulfuric acid (5 ml.) and the

precipitate removed by filtration.   Half the filtrate (10 ml. equivalent to
5 ml. serum) is heated with 1 ml. concentrated sulfuric acid for 20 min.
under reflux.   The precipitate is filtered off, washed, and the filtrate and
washings are extracted with benzene in the same way as a hydrolyzed
urine.   The benzene extract is evaporated to dryness, the residue is dis-
solved in ether, and the solution is washed with alkali and water.   The
ether solution is evaporated and taken up in alcohol for colorimetric
measurement.   The color obtained is yellow-brown, and a correction
factor must be applied.   Values of the order of 15 mg./l. are found.

Tissue ketosteroids are even less amenable to chemical assay.   Sam-
uels (1947) found that steroids added to tissue could be recovered by
extraction and then determined colorimetrically, but the method was not
used to show that tissues contained native steroids.   An interesting
histological method has been described by Camber (1949).   He applied
the device of making arylhydrazones of ketosteroids and coupling these
with diazotized primary aromatic amines to give intensely colored prod-
ucts.   Thus 5-μ sections of adrenal cortical tissue treated with 2-hydroxy-
3-naphthoic acid hydrazide and then with a diazonium salt showed dif-
ferential staining.

III. The Adrenocortical Hormone Group—The Development of Extraction and Chemical Assay Methods

As in the androgen group, attempts to find chemical assay methods
for the adrenocortical hormone group have been directed to finding an
index of adrenocortical function in human subjects, particularly by the
examination of urine.   In the section on assay of the androgen group
reference has already been made to the derivation of the C_{19} steroid
dehydroisoandrosterone from the adrenal cortex and to the characteriza-
tion by Lieberman and Dobriner and their co-workers of compounds with
"labeled structures" in the steroids from extracts of hydrolyzed urine.
In addition, several pregnane and androstane derivatives have been
reported as occurring in the urine of adrenal cortical tumor cases, either
as abnormal constituents or as normal constituents in abnormally large
amount.   Work of this latter type, consisting of fractionation of urinary
steroids and characterization of constituents of the mixture, has led to
nothing which comes within the concept of chemical assay.   For further
information the references already mentioned should be consulted
(Engstrom, 1948; Lieberman and Dobriner, 1948; Mason, 1948).

The chemical assay of "cortin-like" materials is in another category,
and has had as its object the determination by chemical methods of the
group of compounds with the characteristic biological activity of the
adrenal cortex—maintenance of life, salt regulation, and glycogen deposi-

tion.   This began on a basis even less certain than that of androgen assay. A long time elapsed before the first active corticosteroid-like substance was isolated from urine and characterized, and chemical assays were being carried out on material of unknown constitution by not highly specific reactions and even, for some time, without correlation of chemical and biological assays.   This rather adventurous development has, however, been to some extent justified by results.   There are plenty of gaps remaining to be filled by exact knowledge of the materials concerned and of their places in the metabolic scheme, but in rough outline the theoretical foundation seems now at least as sound as that of androgen assay.

The possibility of extracting cortin-like material from urine was recognized a good many years ago (Perla and Marmorsten-Gottesman, 1931), but useful work on urinary assays began when a method of extracting cortin-like material from urine by ethylene dichloride was elaborated by Venning *et al.* (1944).   Talbot *et al.* (1945) showed that the behavior of known adrenocortical steroids in a similar, but more elaborate extraction, and partition between benzene and water justified the assumption that the cortin-like materials in urine were 11-keto- or 11-hydroxy-corticosteroids with a 2-carbon, sugar-like or ketonic chain and possibly a hydroxyl group at C-17, and, further, that the amount of such compounds could be grossly estimated by their reducing action on copper compounds. At a later date, Talbot *et al.* (1947) made extensive use of this method to estimate the excretion of "11-hydroxycorticosteroid-like substances" in normal and abnormal subjects.   The variations observed could be reconciled in all cases with the clinical and pathological observations.

Heard and Sobel (1946) investigated in considerable detail the reaction of reducing steroids with the Folin-Wu phosphomolybdic acid reagent. Thirty-five pure compounds were tested.   The reducing powers and rates of reduction varied greatly, and it was possible to divide the compounds into a number of groups.   The important groups with high reactivity were those of compounds with the primary α-ketol group, $>$CH$\cdot$-CO$\cdot$CH$_2$OH (as in desoxycorticosterone), or $>$C(OH)$\cdot$CO$\cdot$CH$_2$OH (as in Kendall's Compound E), a secondary α-ketol group (cyclic, as in cholestan-2(α)-ol-3-one acetate), or an α,β-unsaturated 3-keto group (as in Δ^4-cholesten-3-one).   In the presence of both α-ketol and α,β-unsaturated 3-keto groups, the reducing power was approximately the sum of the two contributions.

Heard *et al.* (1946) pointed out the close parallel between the chemical structures having reducing power for phosphomolybdic acid and those possessing biological activity.   All the known physiologically active adrenocortical steroids contain the α,β-unsaturated 3-keto grouping and the primary α-ketol side chain at C-17.   It was, therefore, attractive to

assume that the neutral substances in urine, which were extractable by fat solvents and possessed reducing power, were composed, at least in part, of the physiologically active excretory products. Confirmation of this supposition was obtained by correlation of depressed or stimulated adrenocortical function of dogs with chemical assays on extracts of their urine and, further, by comparison of chemical assay and biological assay on urinary extracts from human subjects with normal, depressed, or excessive adrenocortical function. The results of biological assay, by the method of Venning *et al.* (1946), depending on the deposition of glycogen in the liver of the adrenalectomized mouse, were expressed in terms of equivalent weight of Kendall's Compound E. It is notable that the reducing power, expressed as the equivalent weight of desoxy-corticosterone, gave figures some 20–30 times those of the bioassay, e.g., for normal men the reducing power was 1.5 mg. per day and the "cortin"· value 62 μg. per day. Some part of the reducing compounds, perhaps 0.5 mg. per day, were, however, without biological activity. These figures may be compared with the values for normal excretion obtained by the method of Talbot *et al.*, viz., the equivalent of 0.22 mg. per day.

Another method of assay developed from exploratory work by Talbot and Eitingon (1944) and by Fieser *et al.* (1944). Talbot and Eitingon showed that periodic acid would convert certain C_{21} hydroxysteroids to 17-ketosteroids and that determination of the resulting increase in the 17-ketosteroid content of urine extracts provided a basis for an analytical method. Talbot and Eitingon employed the barium chloride or enzymic hydrolysis methods to liberate conjugated metabolites from urine, and emphasized that gentle methods of hydrolysis were essential if C_{21} compounds of the type in question were to survive in the preparation of urine extracts. Fieser *et al.* made similar, independent observations of the oxidizing action of periodic acid. They hoped to be able to isolate, from the non-ketonic fraction of extracts of acid-hydrolyzed urine, characterizable oxidation products, but were unable to do so.

Oxidation of the α-ketol or α-glycol side chains of C_{21} adrenocortical steroids by periodic acid was again investigated by Lowenstein *et al.* (1946), this time with the object of using the formaldehyde formed in the oxidation as a measure of the adrenocortical steroids present. Corcoran and Page (1948) revised the methods first suggested but found inadequate with extracts of urine, and Daughaday *et al.* (1948) also independently devised a workable procedure. In both these the formaldehyde was distilled from the reaction mixture and determined colorimetrically with chromotropic acid. Corcoran and Page expressed results as weight equivalents of desoxycorticosterone and Daughaday *et al.* as arbitrary "cortin" units of similar magnitude. Both methods gave figures for

normal human subjects of the same order as those given by the method of Heard *et al.* (1946) and indicated lowered or increased excretion in cases in which abnormality would be expected from the clinical diagnosis. Corcoran and Page, rather speculatively it may be thought, extended their assay to serum.

Recent publications give some hope of introducing more realism and specificity into assays of urine for corticosteroids. Mason and Sprague (1948) have been the first to succeed in isolating and characterizing a corticosteroid from human urine. A patient with Cushing's syndrome in which the associated diabetes mellitus was unusually severe excreted between 14 and 19 mg. per day of corticosteroid-like material, as assayed by a variant of the Lowenstein *et al.* (1946) method and expressed in terms of 11-dehydrocorticosterone. Chloroform extraction at *p*H 1 followed by solvent partition yielded 17-hydroxycorticosterone in an amount of about 8 mg. per day.

A preliminary publication by Burton *et al.* (1948) deals with the application of paper chromatography to ketosteroids, including corticosteroids. It was easily to be foreseen that partition chromatography would be applied sooner or later to a group of compounds soluble both in water and in fat solvents, and further results will be awaited with interest. Burton *et al.* used 2-ethylhexanol saturated with water as the mobile liquid. Desoxycorticosterone and 11-dehydro-17-hydroxycorticosterone were chromatographed with reproducible results, the positions of the spots being determined with alkaline silver reagent or Jaffe's reaction. The same procedure was applied to chloroform concentrates of adrenal cortex extracts, to neutral ethyl acetate solids of pork adrenal cortex extract, and to neutral extracts of pooled urine of patients in the alarm reaction. Respectively, 4, 3, and 1 mobile components were detected, but no identification was attempted.

What is to be wished for in adrenocortical steroid determination in urines is, undoubtedly, the recognition of significant metabolites. The type of controversy familiar in the androgen field is taking place, and two important workers in the subject (Venning and Browne, 1947) have preferred to adhere to biological methods of assay on the grounds that chemical methods "may include metabolites of adrenal hormones which are not active in the biological assay and give much higher values than the bioassay." Evidence of corticosteroid metabolism is, however, scanty and consists of the detection of metabolites of unidentified precursors (Lieberman *et al.*, 1948), of observations of the low recovery of corticosteroids in the urine when adrenal cortical extract was administered (Venning *et al.* 1944), and of observations on the conversion, in very low yield, of desoxycorticosterone into pregnane-3(α),20(α)-diol in the rabbit,

chimpanzee, and man (Westphal, 1942; Fish *et al.*, 1943; Horwitt *et al.*, 1944), and of the conversion of 11-dehydrocorticosterone into pregnane-3(α),20-diol-11-one (Sprague *et al.*, 1948).

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CHAPTER XVI

Estrogens

By C. W. EMMENS

CONTENTS

I. INTRODUCTION

The great majority of estrogenic preparations in current therapeutic use are pure crystalline substances which do not require biological standardization. The main exceptions are extracts from the urine of pregnant mares, which may contain mixtures of estradiol, equilin, hippulin, and equilenin, possibly together with other unidentified estrogens. These extracts must be standardized by one of the methods described below;

the Allen-Doisy test is usually employed. It will be apparent, therefore, that interest in the biological assay of estrogens centers almost entirely on research and on the determination of the estrogenic activity of clinical material.

Both types of investigation have varying demands, sometimes for extreme sensitivity with no great accuracy, sometimes for the highest attainable accuracy regardless of sensitivity. The range of test methods at present available covers many requirements, although there is a strong tendency for the most sensitive tests to be the least accurate, and the attainment of high sensitivity combined with high accuracy is yet to be reached. However, a word of warning about the precise meaning of the term "accuracy" is necessary. The typical vaginal smear test can give an estimate of the potency of an extract within 80–125% ($P = 0.95$) with about 50 animals per substance under test and 50 on the standard preparation. This, as biological tests go, is fair accuracy, but in fact it only means that repeated tests under the same conditions will give results with the consistency indicated. Different but equally reproducible estimates can be obtained under other conditions, by altering, perhaps, the solvent, the spacing of injections or the number of injections given. The influence of these and other variables is so great that, except under special conditions, it is impossible to attach any precise meaning to the results of an assay unless it is known in advance that the substance under test is in pure form and is identical with the standard preparation in chemical constitution. Yet, if this is known to be the case, it may be superfluous to conduct a biological assay at all, and much of the usefulness of such procedures would seem to be vitiated.

The development of biological methods which are less dependent upon adherence to a rigid assay schedule, or the results of which can be interpreted with less reserve, is only now in progress; and may well be overtaken by accurate and sensitive chemical or physical tests. The continued importance of animal assays depends on the inadequacy of other technics when small quantities of estrogen are to be detected and measured. Chemical isolation of the various estrogens which may be present in an extract is also time-consuming and it may still be preferred, for some purposes, to deal with crude material.

II. TYPES OF ESTROGEN

1. Natural Substances

The natural estrogens which have so far been isolated are all steroids, possessing the cyclopentaphenanthrene ring system:
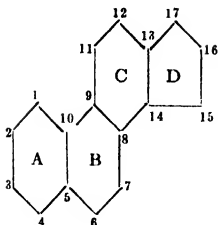
In this, they resemble the androgens and progesterone. Ring A is phenolic, a difference from the other steroid sex hormones, and there is a single methyl group at position 13.

α-Estradiol (estratriene-3,17-diol) is formed by the ovaries and seems to be the main ovarian estrogen. It is not certain, however, that no other estrogen is produced by them. The stereoisomer of the above, *β-estradiol,* is found in the urine of pregnant mares, and is much less potent an estrogen. Some α-estradiol occurs in human urine.

Estrone (estratriene-3-ol-17-one) is found in urine, but has also been isolated from ox adrenal glands (Beall, 1939, 1940) and placentae (Allen *et al.,* 1935; Westerfeld *et al.,* 1938). It is less active than estradiol in most tests, the majority of estimates falling between one-quarter and one-twelfth the activity of α-estradiol. The international unit of estrogenic activity is that of 0.1 μg. of the international standard preparation of estrone.

Estriol (estratriene-3,16,17-triol) is also found in urine and is less active than estrone. The potency reported varies enormously with the test method; values of from 1:1 to 250:1 for the estrone:estriol potency ratio have been found. Both estrone and estriol are formed *in vivo* from estradiol and are excreted in the urine mainly as sulfates and glucuronides (Marrian, 1948).

Equilin (estratetrene-3-ol-17-one) and *equilenin* (estrapentene-3-ol-17-one) differ from estrone in having additional double bonds in positions 7,8; and 6,7 plus 8,9 respectively. They are found in the urine of pregnant mares.

The distribution of estrogens is not confined to vertebrates, or even to the animal kingdom. Donahue (1940) reports their occurrence in marine invertebrates, while many reports have been made of relatively high titers in plant material (cf. Burrows, 1945). The occurrence of a substance presumed to be an estrogen in the locally evolved Dwalganup strain of early-subterranean clover is thought to be responsible for infertility and fetal death in Australian sheep (Bennetts *et al.,* 1946).

Some of the androgens, notably *trans*-androstenediol and 17-methyl-

androstane-17-ol-3-one, cause vaginal cornification in spayed rodents, but are active only in relatively excessive dosage (Parkes, 1935).

2. Synthetic Substances

A large number of compounds not known to occur in nature and sometimes with little structural resemblance to the natural estrogens possesses estrogenic activity. These were first investigated by Dodds and his co-workers, who produced a series of highly potent compounds with no apparent qualitative difference in activity from the natural estrogens. A comprehensive account of the synthetic estrogens is given by Solmssen (1945). Important members of the series are:

Diethylstilbestrol (4,4'-dihydroxy-α,β-diethylstilbene), which is highly active by mouth as well as by injection and has a potency between that of injected estrone and α-estradiol.

Hexestrol (4,4'-dihydroxy-γ,δ-diphenyl-*n*-hexane) and

Dienestrol (4,4'-dihydroxy-γ,δ-diphenyl-β,δ-hexadiene) have similar properties to diethylstilbestrol.

Various halogenated derivatives of triphenylethylene are of interest in having a prolonged action when given by injection, presumably due to their low solubility in body fluids. A diethoxy derivative of triphenyl-bromoethylene (D.B.E.) is said to exert a prolonged action even when given orally (Robson *et al.*, 1942). This is thought to be due to storage of the compound in the fatty tissues of the body.

As a result of the comparison of the local, intravaginal activity of various estrogens with that exhibited on injection Emmens (1941b) concluded that many synthetic estrogens and the "estrogenic" androgens are not themselves estrogenic, but exert their effects after metabolic transformation in the body. Such compounds were called pro-estrogens, and later studies showed the *in vivo* transformation of some of them to true estrogens, detected in the urine (Emmens, 1942, 1943). The importance of these findings when using the intravaginal technic for the assay of estrogens will be discussed below.

3. Esters of Both Natural and Synthetic Substances

The most usual vehicle for the injection of estrogens, whether natural or synthetic, is an oil. When free substances are used, they are absorbed from the site of injection within a day or two and thus exert their effects only transiently. Exceptions to this rule are some of the less soluble synthetics mentioned above. Even large, excessive doses of most estrogens are rapidly absorbed and little prolongation of action is attainable by increasing the amount injected. Those estrogens which are sufficiently soluble in water or saline to be injected in such a vehicle are even

more rapidly lost from the site of injection and excreted or destroyed. For clinical use, some success has been attained with the use of suspensions of crystals in water (Freed and Greenhill, 1941).

The effects of esterification in prolonging the action of steroid hormones was first studied extensively with androgens. Miescher *et al.* (1936) prepared a series of aliphatic esters of testosterone and showed that the prolongation in effect was greater, the longer the aliphatic chain. Subsequent work with esterified estrogens gave similar results. Parkes (1937) investigated the esters of estradiol, using the response of the breast feathers of the Brown Leghorn capon as a convenient index. These feathers grow at about 2 mm./day in the strain used by Parkes, and the duration of action of the various esters can be measured by the length of the zone of fawn (female) color produced on the otherwise black feather. The minimum dose required to produce an estrogenic effect rises with the length of the aliphatic chain, or chains. The effect of a single injection of 1 mg. of free estradiol lasted in Parkes' tests for about 2 days, with no obvious prolongation if higher doses were given. A single injection of 1 mg. of estradiol diacetate feminized the plumage for 6 days, of the 3-benzoate-17-acetate for 19 days, and of the monobenzoate for about 12 days. Deanesly and Parkes (1937) injected estradiol monobenzoate intravenously and found that its action was not prolonged under that condition, thus indicating that the mechanism by which esterification exerts its characteristic effect is by delaying absorption from the site of injection.

In later studies, Dodds *et al.* (1938) and Emmens (1939b) showed similar prolongation of action in rodents, using the mono- and diesters of both natural and synthetic estrogens. The latter author also showed that there is no prolongation of the action of esters when given by mouth, beyond that seen with corresponding doses of the free compounds, with which it is possible to produce vaginal cornification for 3–4 days if large doses are given. He also demonstrated a sharp rise in minimal effective dose as the ester chain is lengthened, so that injected diethylstilbestrol dipalmitate must be given in 500 times the dose required with the free compound. It then lasts for about 18 days in effecting vaginal cornification.

Emmens (1941) showed that esterification is almost, or perhaps completely, without effect when estrogens are given intravaginally. Thus, the median effective dose of estrone, when given intravaginally in an Allen-Doisy test, using two applications, was 0.00029 μg.; that of estrone butyrate was 0.00022 μg.; and of estrone caproate, 0.00021 μg. (as free hormone). Similar results were obtained with estradiol and its monobenzoate, while the higher esters of diethylstilbestrol showed a

slight decrease in activity, the median effective dose rising from 0.00037 μg. of free diethylstilbestrol to 0.00062 μg. of diethylstilbestrol as the dipalmitate.

The significance of these results in considering various methods of assay is obvious. Under such conditions as oral or intravaginal administration, esterification is unimportant and will probably not interfere with potency estimation. Under other conditions, it will completely invalidate any conclusions drawn on the assumption that the free compounds are being assayed.

III. Assays Based on Vaginal Cornification (Allen-Doisy Tests)

1. The Induction of Vaginal Cornification in Rodents

Stockard and Papanicolaou (1917) first reported the occurrence of vaginal cornification in guinea pigs during the estrous cycle. Allen and Doisy (1923) found the same phenomenon in mice and adapted it to the assay of estrogens, using castrated females. Subsequent investigators have used this test, employing sometimes immature but usually castrated female rats or mice, almost to the exclusion of other methods. Our knowledge of the factors affecting estrogen assays is thus largely confined to variants of the Allen-Doisy test, of which there are many. A number of detailed studies have been published on this test, the most comprehensive of which are those by Marrian and Parkes (1929), Emmens (1939a) and Pedersen-Bjergaard (1939). Others which include useful discussion are Allen et al. (1930), de Jongh et al. (1932), and Hain and Robson (1936). All these studies were concerned with orally administered or injected material of diverse origin given to spayed rats or mice.

It seems to have been nowhere clearly stated in the literature that the response of the spayed mouse is easier to classify and score, either quantally or in a series of arbitrary grades, than is that of the spayed rat. Since mice are usually found more convenient to handle and house and also require smaller amounts of active material, it would seem preferable to use the mouse rather than the rat for most purposes. Rats may, however, be found preferable when relatively large amounts of crude material are to be assayed, since they tolerate such injections more readily, or when intravaginal pellets of dried blood or other organic matter are to be used. The latter point has not yet been fully investigated.

2. Spaying the Rat or Mouse

The operation of spaying, or ovariectomy, is best performed in the weanling immature female. A single transverse incision across the midline is made in the skin of the back with the animal under any con-

venient anesthetic. The skin may readily be shifted so that the incision lies first over one ovary and then over the other. A small puncture through the dorsal musculature of just sufficient size to admit the tip of a pair of fine forceps is then made over the site of each ovary, which can usually be seen through the abdominal wall embedded in a small pad of periovarian fat. The fat (not the ovary) is grasped by the forceps and pulled out through the puncture in the body wall. The tip of the uterine horn is then crushed in a pair of artery forceps and the ovary, intact in its capsule, together with the Fallopian tube, is removed, preferably by a single cut with a scalpel or a safety razor blade. The horn of the uterus is released and allowed to slip back into the abdominal cavity. There is usually no bleeding and no necessity to suture the abdominal wall in mice, but a single suture may be found necessary in the rat. On completion of the bilateral operation, the skin incision is closed by 1 or at the most 2 interrupted silk sutures. Aseptic precautions are not necessary, speed and gentle handling are more important.

Failure to remove the Fallopian tube or grasping the ovarian capsule with the forceps involves the danger of incomplete ovariectomy. When the operation is performed as above, incomplete castrates are almost never found. With practice, 30–40 mice or 20–30 rats per hour can be spayed.

3. Preparation of Solutions

Solutions for parenteral or oral administration may be prepared by adding stock solutions in absolute alcohol directly to oil, saline, or distilled water. When this is done, the final concentration of ethyl alcohol in aqueous media should not exceed 2% by volume for oral dosage or 10% for parenteral dosage, and should be kept uniform for all dosage levels. Aqueous solutions for intravaginal administration, or solutions in mixtures of glycerol and water, may contain up to 5% of alcohol. It is not certain that a higher concentration is safe under such conditions, but it may be so.

These solutions must be thoroughly mixed. There is no trouble with aqueous media, but solutions in glycerol and water or in oil should be mixed by prolonged gentle warming on a sand bath or hot plate, with care taken over adjusting the final volume if appreciable evaporation has occurred. With oil solutions it is the alcohol that evaporates, so that in this case it is best to continue warming until no alcohol remains or to replace the evaporated alcohol, using a graduated bottle or flask throughout. Most oils in common use (olive oil, arachis oil, or sesame oil) will not mix with more than about 5% of alcohol.

Alcoholic stock solutions of estrone were found to be stable for many

months, even at 37°C. (Rowlands and Callow, 1935), but care should be taken over cleanliness and to prevent evaporation. Recently, Wilder Smith and Williams (1947) have reported that solutions of dienestrol and diethylstilbestrol at both 1 μg./ml. and 100 μg./ml. lose potency when kept at room temperature either in water or alcohol. Estrone, hexestrol, and estradiol were found to be relatively stable under the same conditions (room temperature for up to 32 weeks), but still showed a slight loss of potency. All five estrogens were little affected if kept in sesame oil at room temperature, or in men's urine in a refrigerator. Aeration, the presence of benzoyl peroxide or hydrogen peroxide increased the rate of inactivation of dienestrol and diethylstilbestrol, while hydroquinone decreased it.

In view of these results, it would seem inadvisable to keep solutions. of any of the estrogens for more than a short period. Emmens (unpublished data) finds that the very dilute solutions used in intravaginal work may be so unstable as to lose potency within a few weeks, but in general agreement with the above has seen no evidence of instability in more concentrated alcoholic or oily solutions of the natural estrogens kept in the cold store for up to 12 months.

4. Preparation of Test Animals

When a colony of rats or mice has been spayed and rested for 10–14 days, it should be primed before use. The sensitivity rises, as does uniformity of response, after a single priming injection of 0.5 μg. to 1/μg. of estrone or diethylstilbestrol. Thereafter, it is not necessary to prime the animals unless a particular group has not been used or has been used but has not responded positively during the past 6 weeks (Emmens, 1939a). Palmer (1941) recommends giving a known overdose of estrogen followed a week later by a threshold dose only. Mice which react positively to both of these doses are used in tests, others are discarded. Palmer finds a greater uniformity in response when this selection procedure is followed.

On the subject of uniformity, it may be noted that Emmens (1939c) did not find more uniform responses when using a highly inbred strain of mice in comparison with a randomly mated stock of albinos. It seems probable that this is not a typical result as the use of inbred animals is frequently an advantage in biological assays.

After preparation of the test animals as above, it is wise to use the whole colony for a determination of the typical dose-response line for injected estrogen in the particular stock of animals, using either international standard estrone or another crystalline estrogen if more convenient. This will check technic and the uniformity and sensitivity

of the animals. The probit-$\log_{10}$ dose line so obtained should have a slope of not less than 5, since there is uniform agreement that a slope of between 5 and 6 is typical in satisfactory assays. The χ^2 test for homogeneity should also indicate no significant departure of group responses from the calculated line.

5. A Typical Allen-Doisy Test

The method employing spayed albino mice as used by Emmens (1939a) for the investigation of crystalline estrogens and of urinary extracts will be described. Solutions for subcutaneous injection are made up in arachis oil and so adjusted that a constant volume of 0.05 to 0.2 ml. is injected on each occasion. It is, however, best to limit the volume to 0.1 ml. if possible, since nut oil is not readily absorbed from the site of injection.

Single injections are given at 10 A.M. on Monday; double injections at 10 A.M. on Monday and Tuesday; quadruple injections at 10 A.M. and 5 P.M. on each of the 2 days. For routine assays, 2 injections are employed. Smears are taken with a fine metal spatula on Wednesday afternoon and discarded, at 11.30 P.M. Wednesday and at 10 A.M. and 4 P.M. on Thursday. The discarded Wednesday smear makes it easier to read the first of the recorded smears. If it is particularly inconvenient to smear on Wednesday night, a smear taken as late as possible may be substituted with some loss of sensitivity. All smears are taken from the dorsal vaginal wall as gently as possible, transferred to a glass slide and stained for 10–15 min. with methylene blue, washed, and scored when dry.

A positive smear contains nucleated or cornified epithelial cells but no leucocytes; a full proestrus smear is thus scored as positive. The response is treated quantally, and is positive if any of the 3 smears from an animal is positive.

Assays using such a method should have at least 2 groups of mice on both the standard and unknown, each group containing not fewer than 20 animals. If fewer animals are placed in the groups, the probability of obtaining 0 or 100% of positive responses and of finishing with very wide or even infinite fiducial limits of error is too high and the procedure only results in waste of time and materials. If 20 animals are used per group and the dose-ratio is about 2:1 in the 2 groups on each substance, a valid assay will usually be performed, with fiducial limits of error between about 70 and 140%, or even less if the dose-response lines from previous assays are available for the estimation of the slope (which does not normally exhibit time-to-time variation). Typical dose-response lines are shown in Figs. 1 and 2.
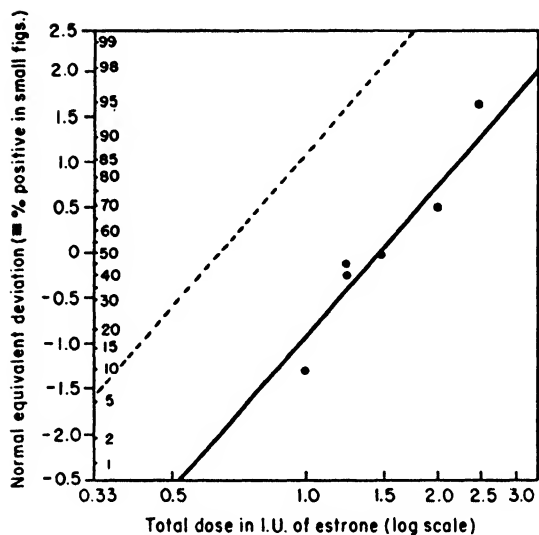
FIG. 1.   A dose-response line for international standard estrone, with 20 mice per group.   The dotted line shows the most extreme shift in the position of the line from its original position, seen in the course of about a year's work with the same mice. (From Emmens, 1939a, reproduced with permission of H. M. Stationery Office, London.)
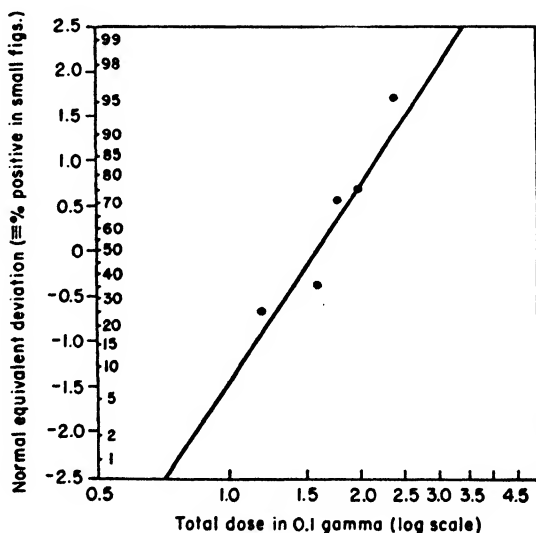
FIG. 2.   A dose-response line for estriol, with 20 mice per group.   (From Emmens, 1939a, reproduced with permission of H. M. Stationery Office, London.)

The most frequent source of trouble is an unexpected change in the sensitivity of the colony of animals. Some degree of control over this can probably be exercised by ensuring that dietary and general environmental conditions are kept constant, and that the animals are in a fully primed state. Despite such measures, however, uncontrolled factors sometimes cause a shift in sensitivity of such a magnitude that an assay is entirely spoiled. It should be noted that, as long as groups are placed on the standard preparation on all occasions, changes in sensitivity are of no importance except in so far as they are a nuisance and lead to wasted effort. They do not affect the precision of tests in which a valid assay has been performed.

IV. Modifications of the Allen-Doisy Test

1. Methods of Administration

Administration may be peroral, subcutaneous, percutaneous, intraperintoneal, intravenous, or intravaginal. The last-named method will be dealt with in full below. Peroral administration may be in oily or aqueous media, aqueous-glycerol mixtures or propylene glycol. With mice, a glass or metal tube about 1.5 mm. in diameter with a blunt, preferably "organ-pipe" outlet is used. It is easier to keep the feeding tube fixed and to thread the mouse onto it than to have both movable. A dose of up to 0.5 ml. may be given on each occasion. An elastic stomach tube may be used for rats.

Subcutaneous administration needs little further comment, except that when using aqueous solutions, a volume of up to 0.5 ml. may also be injected in mice. Percutaneous administration in organic solvents such as alcohol and benzene, applied to the shaved skin, gives as sensitive a test as injection in oil (Emmens, 1941a), but has no particular merits as an assay method except in special circumstances. Again, there is no particular virtue in the intraperitoneal route for most assay purposes, but it has been used by Pincus and Werthessen (1938), who claimed that the potency of some synthetic estrogens is increased 50- to 300-fold in comparison with the subcutaneous route. Results with the rarely used intravenous route in rodents are described by Pedersen-Bjergaard (1939).

The number and spacing of injections varies in different hands from a single initial injection—usually only employed with esterified compounds —to 6 or even 8 spaced out over 2–3 days. With free estrogens in oily solution, little is to be gained by giving more than 2 injections, 1 on each of 2 consecutive days, except in the case of estriol. If aqueous media are used, a minimum of 4 injections is desirable, as the estrogens are

more rapidly absorbed and eliminated than from oil.   The effects of these modifications will be discussed below.

2. Taking and Interpreting Smears

The most convenient and rapid method of smearing is as described above.   Various authors have felt, however, that a gentler technic is desirable and have used cotton wool swabs or have introduced a little saline by means of a pipet and withdrawn it after sucking back and forth a few times.   These are both much more time-consuming, as new material has to be used for each smear.   Even with the swab method Wade and Doisy (1935) report that frequent smearing—3 times daily for 3 or 4 days—produces about 25% of false positives.   This is not a trouble when few smears are taken, but it would always be well to check that the particular routine adopted in a test does not produce responses in· untreated animals.

Some workers omit staining the smears.   This seems to be more successful with the rat than with the mouse, and the methylene blue staining method is so rapid that its omission hardly seems worth while.

The quantal method for scoring is widely used, but some authors employ various grades of response by which they can detect less complete vaginal changes than full cornification or the complete absence of leucocytes.   Thus Mühlbock (1940) uses 7 grades, *a* to *g*, only the latter representing a full reaction.   As none of the authors who have elaborated such systems appears to have subjected his results to statistical investigation, it is not possible to state their exact value.   It would seem, however, that a linear dose-response relationship, necessary to the precise evaluation of results, could be found only by chance when a scoring system of this type is used.   (It would, of course, be necessary to allot numerical values to these grades if they were to be used for assays.)

V. Interpretation of Results with Allen-Doisy Tests

It was pointed out above that the precision with which a particular result may be obtained is misleading, in that quite different results are possible with modifications in technic.   Little in the way of detailed investigations has been reported since the work of Emmens (1939a) and Pedersen-Bjergaard (1939).   Tables I and II, reprinted from Emmens' monograph, illustrate the position as it still is today when injections are given.   They show such wide discrepancies according to the author quoted and the technic used that it is clear that no useful purpose is served by attempting to assay accurately the potency of estrogenic material of unknown (or even known) constitution in terms of, say, international standard estrone.

Studies by both Emmens and Pedersen-Bjergaard of impure extracts from the urine of women and mares revealed similar results. Both were agreed that with the biological methods then available it was impossible to arrive at a trustworthy estimate of the nature or amounts of the estrogens present, and that the two international standards, estrone and estradiol benzoate, can only be used for comparison with preparations

TABLE I

The Ratio of the Potencies of Estriol and Estrone Found by Different Investigators Working with Ovariectomized Rats (From Emmens, 1939a)

Number and nature of injections	Estriol: estrone ratio	Reference
3 aqueous	1:250	Meyer *et al.* (1936)
3 aqueous	1:2	Curtis & Doisy (1931)
4 aqueous	1:2	Cohen & Marrian (1934)
4 aqueous	1:1	Burn & Elphick (1932)
1 oily	1:4.5	Burn & Elphick (1932)
1 oily	1:100	Butenandt & Störmer (1932)
3 oily	1:90	Meyer *et al.* (1936)
? oily	1:2	Marrian (1930).

TABLE II

The Ratio of Potencies of Estradiol and Estrone Found by Different Investigators Using Ovariectomized Rats or Mice (From Emmens, 1939a)

Animal	Nature and number of injections	Estradiol: estrone ratio	Reference
Rats	3 oily	6:1	Schoeller *et al.* (1935)
Rats	6 aqueous	7:1	Schoeller *et al.* (1935)
Mice	3 oily	0.8:1	Schoeller *et al.* (1935)
Mice	6 aqueous	3:1	Schoeller *et al.* (1935)
Rats	1 oily	3:1	David *et al.* (1935)
Mice	1 oily	2:1	David *et al.* (1935)
Mice	3 oily	2:1	David *et al.* (1935)
Mice	6 aqueous	2:1	David *et al.* (1935)
Mice	5 oily	5–10:1	Dirscherl (1936)
Rats	3 oily (α-form)	12:1	Whitman *et al.* (1937)
Rats	3 oily (β-form)	0.3:1	Whitman *et al.* (1937)

known to contain only estrone or estradiol benzoate respectively. Pedersen-Bjergaard found, for example, that an extract of human pregnancy urine assayed at from 158 to 75,900 I.U./g. according to the assay method employed. It can be shown that, in addition to the discrepancies occurring between the different pure estrogens, the situation with urinary extracts is further complicated by the presence of augmenting substances, which will be present in varying amounts according to the extraction technic (Emmens, 1939a).

Some of the discrepancies between different laboratories may be due to the use of impure or incompletely characterized compounds, but this

cannot account for the widely differing results obtained within one laboratory when relatively minor modifications in technic are introduced. These are due to differing rates of utilization and perhaps of metabolism and excretion when test methods are varied. We are thus forced to conclude that assays of impure material by the now classical Allen-Doisy method are only an approximate guide to the amount of estrogen present and may be widely misleading. Furthermore, no deep significance can be attached to the relative potencies found for pure substances, as these are so easily changed by minor variations in technic.

The conditions under which an Allen-Doisy assay may be expected to give the most meaningful results have recently been discussed by Emmens (1947), with particular reference to the assay of urinary steroids. A crude extract, whether made from urine or other sources, will contain a mixture of estrogens and other substances, and the potency it exhibits in any particular test will depend on the method used and the nature of the extract. If there is less than a few milligrams of estrogen in the total extract available, biological assay must at present be used. The ideal biological test method under these conditions would be one which does not differentiate between the various estrogens which may be present, so that the proportions in the mixture would not matter, and is not affected by the presence of extraneous material. Such an assay would give an estimate of the total weight of estrogen in the sample, irrespective of its nature, which would resemble the answer given by the Kober reaction when dealing with large, chemically assayable amounts.

When an injection technic is used, the potencies of the natural estrogens most closely approach one another if absorption is slow, but not too slow. This occurs with hydrolyzed extracts (in which the esters have been broken down) when multiple injections of an oil solution are given, or by the addition of palmitic or other fatty acids (Emmens, 1939a). Augmenting substances of a similar nature to the fatty acids tested are present in some extracts and may so equalize the potencies of the natural estrogens that useful and reasonably consistent results may be obtained by a 2-injection technic. Thus it proves possible, as in the investigations of Callow et al. (1939) and many subsequent workers, to make useful observations of the urinary excretion of estrogens even though the exact significance of the measured potency of extracts is in doubt. When assaying impure extracts biologically by this method, it would thus seem best to have impure extracts really impure, in the hope that the leveling of potency which then tends to occur with the natural estrogens contributes toward attaining a meaningful series of results. Sensitivity is also increased under these conditions, which may be helpful when dealing with low titers of estrogen. However, all such results are of

doubtful fundamental significance, and the modification of the Allen-Doisy test next to be described seems of considerably greater promise.

VI. Intravaginal Allen-Doisy Tests

1. Local Application of Estrogens

Direct local application of androgens to the capon comb was shown by Füssganger (1934) to be a highly efficient method of administration. This was soon followed by the observations of Berger (1935) and Lyons and Templeton (1936), who showed that intravaginal application of estrone and urinary estrogens in the rat requires smaller amounts for positive responses than do other methods. In the rat, oil solutions as used by Berger and others are partially effective, in that about a twelve-fold increase in efficiency is obtainable. In the mouse, irregular responses with oil indicating about a threefold increase were found by Emmens (1939a), who at that time abandoned the method as unreliable. Stadler and Lyons (1938) continuing work with aqueous solutions in the rat, meanwhile showed that positive responses could be obtained with the equivalent of 0.05–1.0 ml. of female urine.

Freud (1939) and Mühlbock (1940) investigated some of the variables associated with the method and the latter author showed that, in the mouse, application in 50% aqueous glycerol gives sensitive and consistent results. He found the median effective doses of estrone, estradiol, and estriol to be 0.00025 μg., 0.0005 μg., and 0.00075 μg. respectively, while the corresponding values for instillation in olive oil were 0.025 μg., 0.0075 μg., and 0.5 μg. Robson and Adler (1940) also made the important observation that the natural and (some) synthetic estrogens act locally without absorption in significant amounts, since a separate vaginal pocket formed from the lower vagina was practically unaffected in spayed mice receiving the compounds in saline in the upper vagina. They also noted that estriol glucuronide is locally active.

In a re-examination of these facts, Emmens (1941b) confirmed Mühlbock's results, obtaining almost identical activities for the three natural estrogens listed above when given in 2 applications in either saline or aqueous glycerol, and discovered the existence of pro-estrogens, mentioned in Section II above. Fortunately for our present purpose, all of the natural estrogens examined, with the exception of some of the weakly estrogenic androgens (which would not, for instance, be found in the phenolic fraction of a urine extract) are true, locally active estrogens. Diethylstilbestrol, hexestrol, and dienestrol are also true estrogens, many other less strongly active synthetics are not, neither is methylethyl-stilbestrol, which is a potent pro-estrogen differing from diethylstilbestrol

only in the substitution of a methyl for one of the 2 ethyl groups in the molecule.

Further results of Emmens showed that esterification is of no importance in intravaginal assays, thus pointing the way to the development of an assay method that (1) shows remarkably little differentiation between the potencies of the natural estrogens and even the more potent synthetics (diethylstilbestrol has a medium effective dose of 0.0004 μg; hexestrol, 0.0009 μg.; dienestrol, 0.0006 μg.) and (2) is unaffected by esterification and the presence of substances acting as augmentors by the subcutaneous route. Unfortunately, Emmens was led from his preliminary dose-response lines to conclude that these are dissimilar for the three natural estrogens, and that the intravaginal route is probably not adaptable for precise assays of unknown estrogens against a pure standard. This conclusion has remained unchallenged, except by its author, who now believes that a review of the problem is due (Emmens, 1947) and has obtained evidence for the similarity of the three dose-response lines under more rigid conditions of testing (Emmens, 1950).

It would seem that the activity of the most potent estrogens is possibly the same; that they are equivalent or practically equivalent in action on a molar basis, and that a 2- or 3-application intravaginal technic allows of something approaching complete utilization of the material applied. Once this were established, if true, and a technic evolved for taking advantage of it, the biological assay of natural estrogens would be on a very much better footing.

Albrieux (1941a, b) investigated the assay of estrogens in blood by the insertion of pellets of dried material into the vaginae of ovariectomized rats. He obtained positive responses with the blood of non-pregnant women, and also concluded that the corpuscles contain most of the estrogen. The method was confirmed by Krichesky and Glass (1947). This further adaptation of the intravaginal method has yet to be placed on a fully quantitative basis and to be explored with mice or other species.

2. Assays with Aqueous Media

It is perhaps rather premature to describe a detailed method for intravaginal assays in the strict sense of the term "assay," since relatively little accurate work has been done. However, the modifications needed do not involve any alteration in principle, and results are worked out just as with any quantal assay; all that is needed is a description of the changes in technic and timing which are necessary.

Applications in 50% aqueous glycerol are preferable to those in saline or distilled water, mainly because they are better retained in the vagina. The mouse vagina cannot hold more than about 0.02 ml. at a time, and

for routine work 0.01 ml. is best. This is delivered by a micrometer syringe, such as the "Agla," using a blunt-ended wide-bore needle with a side aperture near to the tip, withdrawing the needle slightly as the application is made so as to leave a sufficient space for the fluid. Applications are made at the same times as injections in the more usual test, but smears must be taken earlier, since cornification occurs about a day earlier when the estrogen is applied locally. A preliminary "clearing" smear is therefore taken on Tuesday evening, if the test starts on Monday, and others on Wednesday morning and evening, and on Thursday morning, after which the smear, if positive before, usually reverts to the diestrous type. Staining and scoring are exactly as before, with the proviso that more than about 90% of positive responses is only found with a relative overdose of estrogen in the author's experience, and it is best to confine doses to a range expected to produce responses which fall short of this level.

There is no published evidence as to the need for priming in intravaginal assays, but again, in the author's experience, this is necessary as with other methods. No very accurate data can be given on the expected precision of intravaginal assays. Emmens (1950) finds a mean slope of about 2.7 in contrast to a slope of 5 to 6, characteristic of injection assays. More animals will therefore be needed per group for precision comparable with a normal Allen-Doisy test. With a slope of 3, a 4-point assay, using 20 animals per group (80 in all) will be expected to give minimal limits of error ($P = 0.95$) of 62–162%.

3. Assays with Intravaginal Pellets

This technic has only been described so far for the assay of blood estrogens, but it is clearly adaptable to other purposes. The method needs further analysis, in particular relating to the use of a standard, presumably best administered in a similar pellet form by means of a suitable excipient. As that has not yet been done, this report will be confined to a description of the preparation of dried blood pellets and an indication of the responses obtained. Albrieux (1941a) compared the effects produced by his blood pellets, containing 10% of sesame oil as a "binder," with those of international standard estrone in sesame oil. The rationale of this is not apparent, as estrogen in an aqueous medium is much more effective locally than in oil, and the exact conditions of absorption from an oil-bound blood tablet are unknown.

By Albrieux's method, whole blood is placed in a cellophane tube and suspended in front of an electric fan at room temperature. It dries in a few hours and is then mixed with 10% of sesame oil and hammered into small pellets weighing from 40 to 80 mg. in a tablet-making machine as

described by Mark and Biskind (1940) or Hartman (1940). Separate pellets made from serum or cells can be prepared by centrifuging the blood. The pellets are inserted into the vagina with a trocar, and are said to be completely dissolved in 24 hr. By this method, up to 560 rat units per 100 ml. of blood were found in non-pregnant women, while blood from a surgical castrate caused no vaginal cornification, and it was concluded that no foreign-body reaction occurs.

Krichesky and Glass (1947) dried rabbit or human blood in a vacuum desiccator over calcium chloride, ground it to a fine powder, and then prepared pellets as above. Each pellet was then bisected and one part placed in the vagina on each of 2 consecutive days, thus simulating a 2-application intravaginal technic. It was found that 10 ml. of either rabbit or human blood, when dry, weighed 2.0–2.2 g. Their results seem to agree in general with Albrieux's, except that in their experience positive smears were obtained with the blood from ovariectomized rabbits and women, at a level which indicated about half the estrogen content of intact females in either species. It is impossible to decide at this stage whether Krichesky and Glass were dealing with a non-specific reaction or whether they have detected estrogen in the blood of spayed females.

VII. ASSAYS BASED ON UTERINE WEIGHT

1. Four-Day Tests

Bülbring and Burn (1935) described a test in which young female rats were spayed at about 40 g. and injected 2 days later for 4 consecutive days with estrogen in olive oil. On the eighth day from operation, the uteri were excised, fixed, and weighed, after drying with filter paper. This and similar tests are cumbersome and take rather a long time to complete. Later investigators have successfully used immature intact rats, mice or guinea pigs in shorter tests.

Using the immature, 22 to 23-day-old albino rat, weighing 34–39 g., Lauson et al. (1939) developed a 4-day test. The animals are injected twice daily for 3 days with 0.5 ml. of an aqueous solution of estrogen and killed on the fourth day (72–75 hr. after the first injection). The uteri were separated from the vaginae by cutting through the cervix, the surrounding tissue was stripped off, and the utero-tubal junction severed. They were then weighed fresh, after pressing out the intra-uterine fluid on moistened blotting paper, using a Roller-Smith torsion balance. Weights of uteri before expressing the intra-uterine fluid were found to be valueless. Results with estradiol and estrone are shown in Table III. Vaginal opening was also noted and is shown in the table. Estriol was also investigated, and was found to produce uteri with a maximal mean

weight of about 50 mg., in contrast to the 90- to 100-mg. levels found with the higher doses of estradiol and estrone. This confirms the finding of Dorfman et al. (1935), who used the difference as a means of distinguishing estriol from estrone. On the ascending portion of the curve, the approximate equivalents of 1 μg. of estradiol are 6 μg. of estriol and 20 μg. of estrone. The greater potency of estriol as compared with estrone is a peculiarity of this type as assay.

TABLE III

Uterine and Vaginal Response to Estradiol and Estrone (From Lauson et al., 1939)

Estradiol					Estrone				
No. of rats	Total dose μg.	Mean uterine wt. mg.	Std. dev.	No. of vaginas open	No. of rats	Total dose μg.	Mean uterine wt. mg.	Std. dev.	No. of vaginas open
55	Control	19.6	2.63	0	55	Control	19.6	2.63	0
13	0.025	27.5	3.20	0	15	1.0	41.1	9.06	0
14	0.05	36.3	5.90	0	15	2.0	52.7	10.93	0
14	0.10	50.0	5.80	0	15	3.0	60.3	8.43	2
14	0.15	62.3	6.97	1	15	4.0	77.6	14.41	8
14	0.20	67.4	5.43	9	15	6.0	91.6	9.58	12
14	0.30	88.1	6.99	13	15	8.0	101.7	9.98	15
14	0.40	92.8	8.57	14	15	10.0	105.4	10.40	15
14	0.75	90.0	9.30	14					

The dose-response curves are of a type which lend themselves to an assay using uterine weight and log dose, with groups on the standard and unknown, but this method was not employed by the authors. Later, check assays with pure estrogens were made against the previously determined curves, with from 4 to 26 rats/dose. These gave a wide splay of errors, as would be expected from the conditions of testing, only 21 out of 25 assays of estrone and 25 out of 34 assays of estradiol coming within $\pm 50\%$ of the correct figure. From the data supplied by Lauson et al., it is however possible to calculate approximately the precision an assay of any given type would have. The standard deviations shown in Table III, although rather higher on the average for estrone, are fairly constant for any one substance. An assay of estrone against estradiol would have an average unit variance of about 75, and a slope of about 60 (uterine weight increase per tenfold dose increase). If a total of 40 animals were used in a 4-point assay, the expected minimal limits or error are therefore approximately $(P = 0.95)$ 81–124%, a reasonably low error. The fiducial limits of error, if not founded on a well-established slope from other similar assays, would be wider. It should be noted, however, that the marked dissimilarity in dose-response curves for estradiol and estriol would preclude the assay of one in terms of the other,

and presumably of urine or other extracts in terms of a crystalline standard.

The use of oil solutions was also investigated, and it was found that rats receiving a daily injection in 0.2 ml. of oil (type not stated) gave uterine weights similar, dose for dose, to those shown above. The authors also noted changes in sensitivity of the animals from time to time, a strong pointer, if any be necessary, to the need for the simultaneous assay of unknown against standard on each occasion.

The uterine reaction to gonadotropin in the mouse has been studied by Hamburger and Pedersen-Bjergaard (1937), Levin and Tyndale (1937), Evans *et al.* (1940) and von Haam (1940). The mouse is more sensitive than the rat, as in Allen-Doisy tests, and in gonadotropin assays it gives a more uniform response. Evans *et al.* (1941) therefore tried the mouse uterine assay for estrogens (the gonadotropin assay utilizes· the production of estrogens by the stimulated gonads). Their figures suggest that the mouse assay is more accurate than the assay using rats as described by Lauson *et al.*, but this is an inference from the scatter of points about dose-response lines they present made from tests with a similar injection and time schedule to that just described. Unfortunately, Evans *et al.* do not give sufficient details for a critical appraisal of their results, and no useful estimate can be made of the probable accuracy of tests.

The mouse uterine weight curves also seem to show differences in shape between the natural estrogens, but it is not certain, from the data presented, that this is not merely a reflection of differences in potency, as no flat maxima are shown for estriol and estrone. The order of activity, in contrast to the rat uterine tests, is estradiol, estrone, estriol (the weakest).

Uterine weight can be increased by androgens and progestogens, in considerably higher doses than are needed of the commonly encountered estrogens. These tests are therefore not completely specific, although the presence of sufficient androgenic or progestational material to augment the effects of estrogen seems unlikely, even in unfractionated urine extracts. Thus, Evans *et al.* (1941) found that the metrotropic activity of androsterone is less than one ten-thousandth of that of estrone, and in tests in which androsterone and dehydro*iso*androsterone were added to injections of estrone in ratios likely to be found in human urine, Evans and his colleagues found no significant effect.

2. Astwood's Six-Hour Test

Astwood (1938a) studied the early effects of estrogen on the uterus of the immature rat, and showed that a rapid increase in weight occurs

during the first few hours after an injection.   This increase is due almost
entirely to the accumulation of water, as is shown in Fig. 3.   The increase
has been made the basis of a 6-hr. test for estrogenic activity (Astwood,
1938b), at which time the early weight increase is at a maximum.

In Astwood's original work, all doses were given in a single injection
of 0.1 ml. sesame oil under the skin of the back.   Female albino rats
21–23 days old were used, weighing 25–49 g., mean 36 g.   After 6 hr.,
the animals were killed with chloroform, and the uteri were dissected
out by cutting at the utero-tubal junctions, stripping off the mesometria,
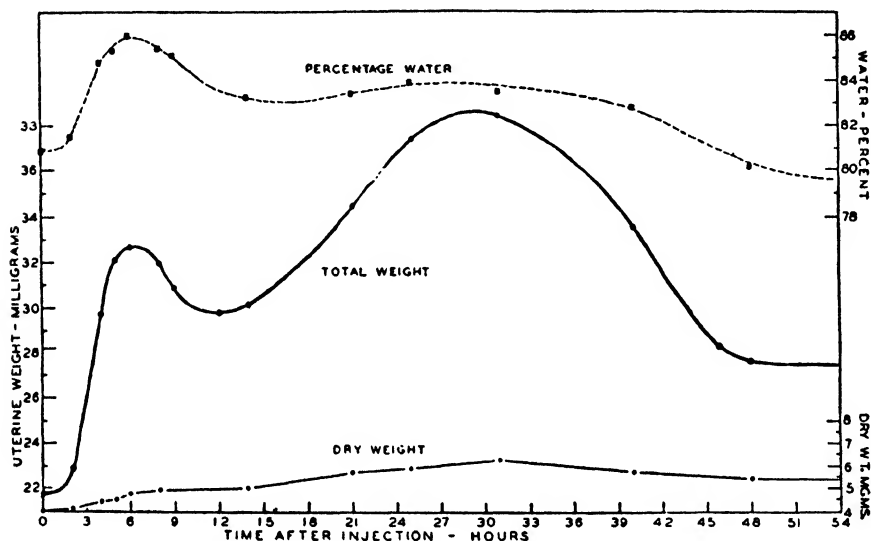
FIG. 3.   Changes in % water, total weight and dry weight of immature rat uteri
during 48 hr. following a single subcutaneous injection of 0.1μg. of estradiol in oil.
The curve represents means figures from 370 rats.   (From Astwood, 1938b.)

and trimming the vagina from its attachment to the cervix.   After
blotting on absorbent paper, the uteri were quickly weighed on a damped
analytical balance.   Water determinations were made by desiccating
the weighed uteri in an oven at 110°C.   Astwood then "partially cor-
rected" for variation in sizes of rats by expressing all uterine weights in
terms of an animal of a standard body weight of 36 g.   He noted that the
correction tended to over-correct for animals at the extremes of the
normal range.   The crude data were then further treated in producing
assay curved by using the percentage increase in uterine weight as a
criterion of response, but as this was apparently done on the basis of a
single group of controls, common to all assays, it would not be a source

of time-to-time variation and would presumably leave the nature and accuracy of the dose-response line unaffected.

Estradiol and estrone were shown to have similar dose-response curves, that for estradiol is shown in Fig. 4. This is a standard curve based on data accumulated over a period, with standard errors of the means shown graphically. Astwood does not give his data in such a form as to allow of calculations relating to the probable errors of assays, and one can only judge roughly from the figures and data given that the method is probably
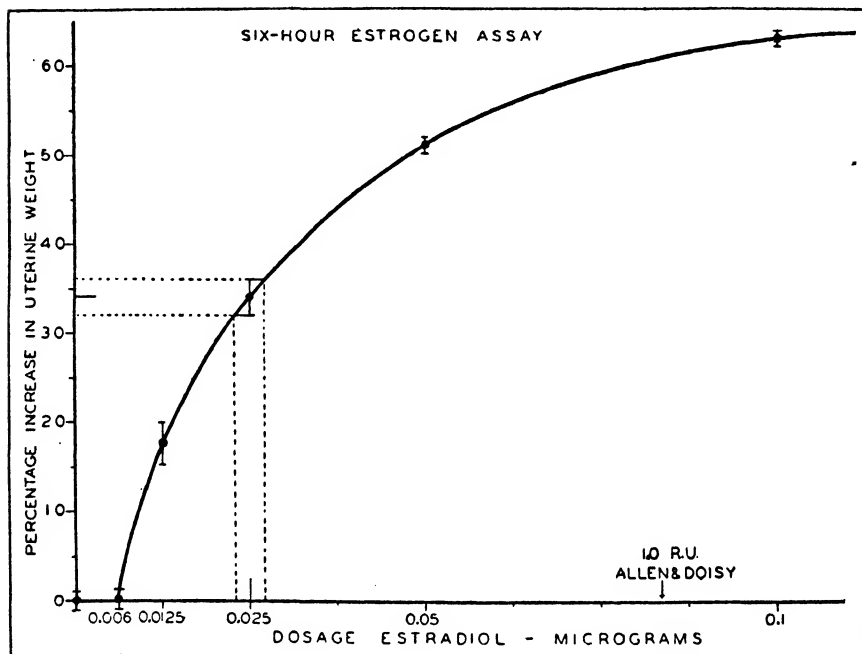
FIG. 4. Standardization curve from 300 rats in the Astwood 6-hr. test. (From Astwood, 1938b.)

fairly accurate. No subsequent critical applications of the method seem to have been made.

In the hands of its author, the Astwood test covered a range of doses of from 0.006 to 0.1 μg. of estradiol, and from about 0.07 to 1.2 μg. of estrone, which has one-twelfth the potency of estradiol. Four assays of the potency of estrone in terms of estradiol, each on only 5–7 animals, made by reading from the standard curve, gave estimates of 12.7, 12.2, 11.4, and 13.6, mean 12.4 μg. estrone = 1 μg. estradiol. The wide dosage range over which estimates may be made is a recommendation, although a more orthodox method of conducting the assay is to be desired. It

seems probable that a 4-point assay using crude uterine weight as the criterion of response, with covariance for body weight correction, instead of the arbitrary method described above, would give reasonably precise estimates of potency. It may be noted that, from Astwood's data, the log dose-response relationship is linear and adaptable to such an assay.

VIII. ASSAYS BASED ON VAGINAL OPENING

Tabulated data such as those in Table III sometimes include observations of the incidence of vaginal opening, made incidentally and not used for the estimation of potency. From these results of Lauson *et al.* (1939), however, it can be seen that vaginal opening may be a sensitive and accurate index of estrogenic activity. The slope of the probit-log dose line is in this instance 7.8 approximately, indicating that a test based on vaginal opening might have a greater accuracy than an Allen-Doisy test (slope, 5 to 6) with the same number of animals. The advantage of such an assay method is, of course, that the animals need not be killed at the end of the test period and are available for other purposes.

Interest in these tests has been aroused by the reports of Hartman and his colleagues on the production of vaginal opening in immature rodents following the local injection of small quantities of estrogen. Hartman and Littrell (1945) produced vaginal opening with small doses of estrogen (probably incorrectly stated to be 0.0005 mg. of estradiol) or of 0.02 ml. of finger blood from a woman, but not from a man. This was injected locally around the region of future vaginal opening in the 21-day-old female rat and was followed in 4–5 days by premature vaginal opening. Recognizable changes were said to follow within 1 day, the first reaction being a crescent-shaped transverse dimpling at the future site of opening, followed by pinpoint punctures with oozing of fluid. Large doses caused complete opening within 24 hr.

The same method was then explored in the guinea pig (Littrell *et al.*, 1946a, b), with similar results, using either immature or castrate females. A full account of the technic is given by Hartman *et al.* (1946). Injection is made with a short beveled needle just beneath the skin on each side of the vulva, one half of the total dose being injected at each site. The needle is inserted at the tip of each horn of the crescentic depression extending across the perineum and is directed towards the midline underneath the horn of the crescent. When the closure membrane of the immature 200-g. guinea pig is intact, the vaginal area is dry and pallid. Within a few hours after the injection of estrogen, the vulval and circumanal regions appear congested, and if observations are made at approximately hourly intervals, the following 5 stages, as described by Hartman *et al.* (1946), may be detected.

"*Line formation.* This consists of an intense blanching, somewhat like the stigma of a maturing egg follicle in the hen. It usually forms in the midportion of the vagina crescent.

"*Dimpling.* There appears as an indentation much like the infolding of the dorsal lip of the blastopore of the frog egg. Since line formation may be quite transitory, dimpling often seems to be the first stage in the reaction. It is followed either by stage 3 or stage 4.
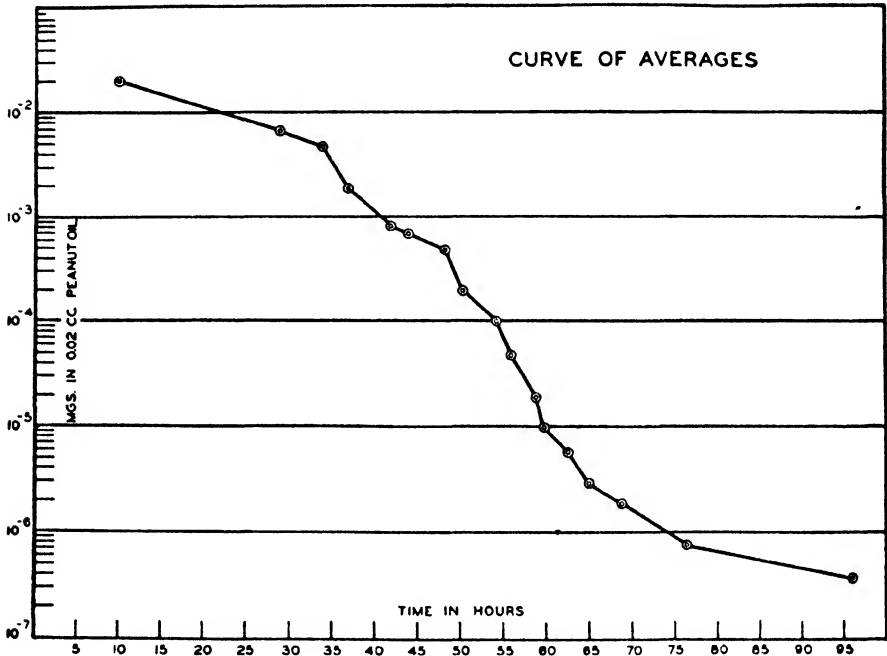
FIG. 5. Dose-response curve for the weanling guinea pig test using estradiol dipropionate in peanut oil. (From Hartman *et al.*, 1946.)

"*Pinpoint formation.* A pinpoint opening may occur at any place in the vaginal crescent. Although very small, it is a definite opening into which an applicator may be inserted. It may increase in size and become a slit or remain a pinpoint opening for several hours. Often the entire crescent may then dimple and complete opening follow.

"*Slit formation.* A slit-like break may follow line or pinpoint formation. The slit may be present from 3 to 18 hours before the vagina opens completely.

"*Complete opening*, with secretion of mucus and cornification of the mucosa.

"Lest it seem over-meticulous to differentiate between these stages

it should be said that this is necessary because the process of opening may be arrested at one of these stages whenever the quantity of the injected hormone is insufficient to carry the process to completion."

The time which elapsed between injection and the opening of the vagina has been studied as a basis of an assay method. This varied between 10 and 96 hr. over a dose range of from 2×10^{-2} to 4×10^{-7} mg. of estradiol dipropionate. A dose of 2×10^{-7} mg. did not cause vaginal opening in the single animal receiving it. The combined dose-response curve obtained with all the animals used is shown in Fig. 5, most points on which are derived from only 1 to 3 observations. The central portion has a very gentle slope, but in the absence of sufficient data from which to form an estimate of the variance of individual responses, it is difficult to hazard an opinion about the probable accuracy of assays.

Lloyd *et al.* (1946) have used the Hartman test with immature female rats and report that it is insensitive to injections of whole blood or serum from women in various phases of the menstrual cycle, and to the urine of various patients. In a further study of the effects of crystalline estrogens, they also found the lowest effective dose of estrone to be 2.5×10^{-4} mg. and of estriol, 2.0×10^{-4} mg. They further cast doubt on the reproducibility of results. Priming the rats with a small subcutaneous injection of estrone was found to increase sensitivity slightly, but variability remained too high for the method to be used as an assay.

Pending further studies, it is therefore impossible to make any specific recommendations about this suggested assay method.

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Hormones of the Corpus Luteum

By C. W. EMMENS

CONTENTS

I. PROGESTOGENS

1. Introduction

In contrast to the great variety of natural and synthetic compounds possessing estrogenic activity, the only known naturally occurring compound with full progestational activity is progesterone, while very few other compounds have been discovered which have progestational effects. As with many other steroid hormones, progesterone can be produced in pure crystalline form and is normally so prepared for clinical use. Its excretion product in man is the hormonally inert substance, pregnanediol, the chemical estimation of which is described in Chapter XVIII. There is thus little need for the clinical estimation of progesterone, or for its

biological standardization, and the attempted assay of the compound has been largely confined to other types of research. This is perhaps fortunate, as there is to date no very accurate or simple method available, and no record in the literature known to the present author of the biological assay of progesterone employing modern statistical methods. The account which follows is therefore a résumé of methods of detecting progestational activity which have been put forward as suitable for assay, rather than of adequately tested assay technics in the full sense of the term, and it has in no instance been possible to estimate the limits of error of a test method.

Progesterone was discovered by Corner (1928), who described its effects in causing proliferation of the uterine endometrium. At about the same time, Weichert (1928) and Goldstein and Tatelbaum (1929) described the sensitization of the rodent uterus to the formation of deciduomata by ovarian extracts, an effect soon recognized as due to the action of "progestin." Various other actions attributed to progesterone, such as mucification of the rodent vagina, relaxation of the pubic symphysis and reversal or loss of the response of the isolated uterus to pituitrin have since been shown to be non-specific or due to contaminants. Nearly all the research leading to the isolation of progesterone utilized modifications of Corner and Allen's technic, using proliferation of the uterine endometrium of the estrogen-primed rabbit as a test object. Owing to the employment of arbitrary, although well-defined grades instead of a continuous measure of response and to the relatively few animals that could be used in a test, this method could not be adapted for the purpose of accurate measurement, and it is only recently that other methods, such as that of Hooker and Forbes (1947), promise a more practicable type of assay.

2. Types of Progestogen

A. PROGESTERONE

Progesterone is Δ^4-androstene-17(β)-[1-ketoethyl]-3-one:
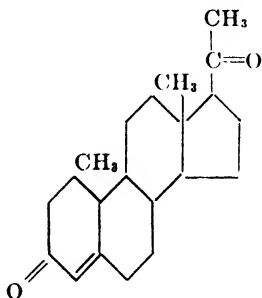
and is thus related to the androgens. Its excretion product in the urine is pregnanediol sodium glucuronidate:
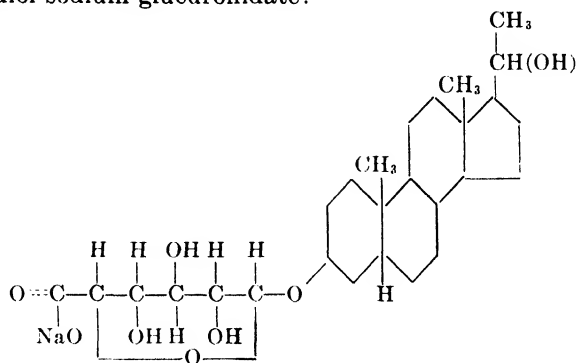
Pregnanediol has no known hormonal action, but is an anesthetic, and therefore cannot be said to be biologically inactive. Not all of the progesterone in the body is converted to pregnanediol, but changes in the excretion of this compound appear to follow changes in the production of progesterone closely.

Progesterone has been isolated only from the corpus luteum. It is reported to be present in follicular fluid (Hooker and Forbes, 1947) and presumed (with fair certainty) to be formed by the placenta of some of the equids, primates, and probably many other species. The international unit of progestational activity is that of 1 mg. of the international standard preparation of progesterone.

B. SYNTHETIC SUBSTANCES

Ethinyl testosterone (pregneninolone, anhydro-hydroxyprogesterone, Δ^4-androstene-17(β)-ethinyl-17(α)-ol-3-one) is the most potent of the few synthetic progestogens. By the McPhail (1934) test in rabbits it has about one-tenth the activity of progesterone and is as active by mouth as it is by injection (Emmens and Parkes, 1939). It is of interest in being,

Ethinyl testosterone

Ethinyl androstenediol
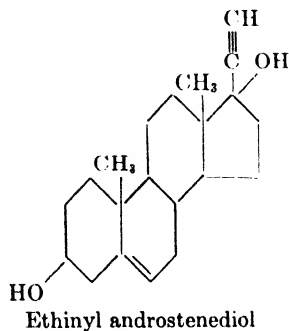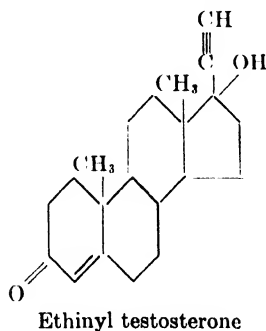
in addition, both a weak androgen and a weak estrogen and in possessing metrotropic activity. These other actions are so weak as to be unimportant clinically, although its estrogenic potency in the rat is sufficient to allow of progestational changes in the absence of prior treatment with estrogen.

Ethinyl androstenediol (Δ^5-androstene-17(β)-ethinyl-3(β),17(α)-diol) has about the half of the progestational activity of the foregoing, and is also active by mouth. It is said to be a weaker androgen.

These compounds are both weakly soluble in oil, but more soluble in propylene glycol, and when injected may remain at the site of injection for some period.

3. Tests Based on Progestational Proliferation in the Rabbit

A. THE CORNER AND ALLEN (1929) TEST

This test, the basis of tests using progestational proliferation in rabbits, is only of historic interest. Previously isolated adult does were mated and insemination proved by the finding of spermatozoa in vaginal smears. The does were again isolated and about 18 hr. after mating examined at laparotomy for ruptured follicles. If ovulation had occurred, bilateral ovariectomy was performed, and a piece of the left horn of the uterus was excised. This test animal was then injected for 5 days with the extract under examination, one subcutaneous injection being given per day. On the sixth day, it was killed, and the genital tract was removed. The uterus and Fallopian tubes were washed out to obtain the segmenting ova, if present, and the uterine cornua were sectioned. The authors noted that responses were obtained even in the absence of ovulation, but felt that mated does should be used for preference. (They also wished to study the embryos.)

The response was judged as an all-or-none reaction, but full proliferation indistinguishable from a normal 6-day pregnancy was demanded. This is now known to require between 1 mg. and 2 mg. of progesterone in the average rabbit.

In trials with immature rabbits, Corner and Allen found very variable results, and discontinued using them. Clauberg (1939a-d), however, found that this was due to the variable state of the immature rabbit (which would normally have produced insufficient estrogen to sensitize the uterus to progesterone) and used either intact or ovariectomized immature rabbits between 600 and 800 g. in weight, primed 8 days prior to the test with 10 "mouse units" of estrin per day. Allen (1930) also reported regular results with estrogen-primed immature rabbits, but did not use them for assays.
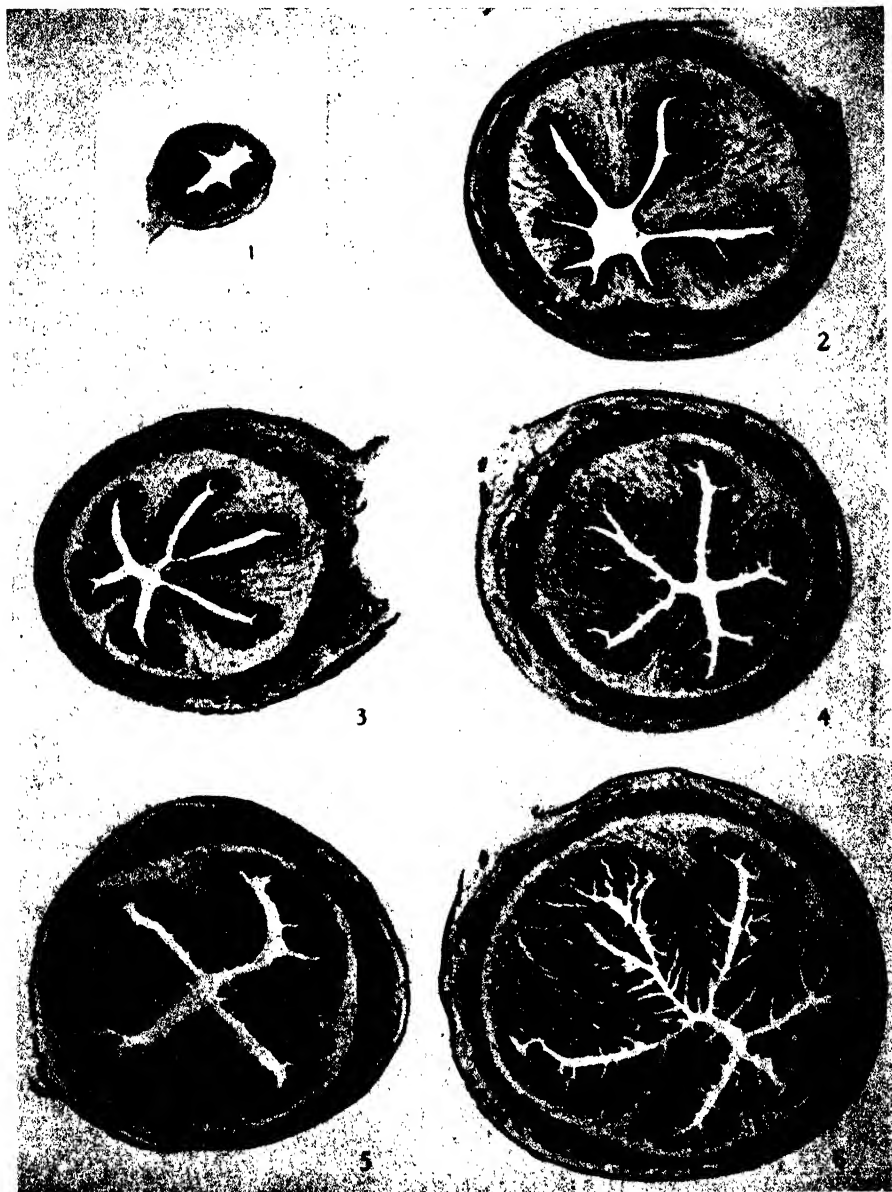
FIGS. 1-6. The standard scale of progestational proliferation in the McPhail test. Fig. 1, no treatment; Fig. 2, estrogen alone; Figs. 3-6, reactions 1, 2, 3, and 4 respectively following the administration of progesterone. (From McPhail, 1934.)

B. THE McPHAIL (1934) TEST

In 1934, McPhail published the results of a re-examination of these methods of testing for progestogens and considerably clarified the situation. He examined many of the variables of the test systematically and showed that a method similar to that proposed by Allen (1930) was suitable for assessing degrees of proliferation. McPhail's 5 stages, together with a control, are shown in Figs. 1-6, which are the type specimens used throughout his tests. The stages shown in Figs. 3-6 inclusive represent scores of 1, 2, 3, and 4, and were compared with an "unknown" by a
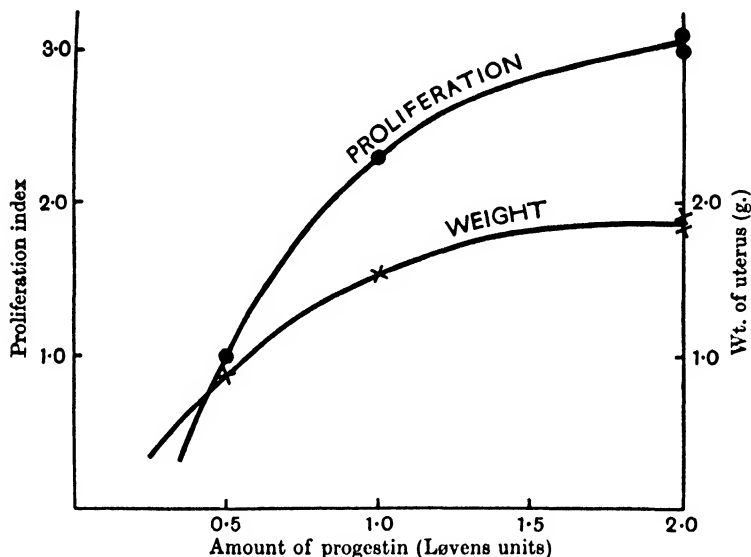
FIG. 7.   Proliferation index and uterine weight of rabbits receiving different doses of progestogen over 5 days.   (From McPhail, 1934.)

method of simultaneous projection. For histological examination, the uterus was fixed in Bouin's fluid and stained with hematoxylin and eosin. Sections were cut at 10 μ.

From his results, McPhail concluded that the full estrus condition could be produced in 6 days with 150 I.U. of estrogen, more estrogen or a shorter period of injections being undesirable. He also found that, under these conditions, injection of progesterone for 3-5 days gave optimal responses, with a sharp decrease in response if continued too long. Thus 1 Løvens unit of progestin given over 5 days elicited a greater response than 2 units spread over 10 days, the daily dose being kept constant. He defined his unit of progestin as that amount which given intramuscularly in oil over 5 days, following the injection of 150 I.U. of estrogen over 6 days, causes an average proliferation of 2 in a group of

immature rabbits weighing 750–950 g.   (This is about 0.75 mg. of progesterone.)   He also remarked that even groups of 5 rabbits involve considerable labor in routine assay, although the variability is such that larger groups seemed desirable.   No attempts were made to compare one substance with another, used as a standard, and no indication of variability was given.   Figure 7 shows McPhail's dose-response curve with 5 rabbits per group.

C. LOCAL PROGESTATIONAL TESTS

Mussio-Fournier et al. (1938) reported greater sensitivity of the rabbit uterus to progesterone injected directly into a uterine segment, obtaining a "minimal response" with 0.1 mg.   McGinty et al. (1939) and Haskins (1939, 1941) found responses with as little as 0.25 μg., and Halvorsen (1944) quoted by Hooker and Forbes (1947) obtained responses in 4 out of 10 animals with only 0.0025 μg. of progesterone.

McGinty et al. (1939) primed 950-g. immature rabbits as recommended by McPhail and exposed the uterus at the end of the priming period.   An upper middle segment of each horn, 3–4 cm. long, was then ligated, the upper ligature was tied tightly and the lower ligature left loose, disturbance to the blood supply being avoided.   Each horn was then sectioned below the lower ligature and crystalline progesterone dissolved in 0.1 ml. of peanut oil or lanolin introduced into the lumen of one horn with a blunt needle on a small syringe.   As the injection was completed, the lower ligature was drawn tight.   The procedure was then repeated with the opposite horn, injecting only the vehicle as a control. Seventy-two hours later the rabbits were killed and the uteri were sectioned, portions below the isolated segments being taken as well as the segments themselves.   The degree of reaction was scored as by McPhail (1934).   In no case did any but the treated segments containing progesterone exhibit proliferation.

The results are summarized in Table I, from which it can be seen that a reasonably consistent response was obtained with the lower doses on peanut oil, with the exception of the lowest dose.   This may, however, be only an example of secular variation in response.

Haskins' (1939) results were similar, and were a repetition of McGinty and his colleagues' work.   He also reported positive responses with 0.2 ml. of serum from pregnant guinea pigs, with no reactions to serum from non-pregnant animals.

It should be noted that progestational responses of the above type may be obtained by injected androgens and possibly other steroids.   The proliferation test is therefore not specific, except that no other natural steroid than progesterone seems to produce a full grade 4 response.

TABLE I

Responses to Intra-Uterine Progesterone (Adapted from McGinty et al., 1939). Half Values Were Scored by the Authors as e.g. 2–3

Dose of progesterone in μg.	Nos. of obs.	Responses obtained with:		Nos. of obs.		
		Peanut oil			Lanolin	
		Mean	Range		Mean	Range
20	2	3.5	3½	2	3.5	3–4
10	10	2.7	½–4	2	3.8	3½–4
5	6	2.3	2–3	6	3.3	2–4
1	6	2.2	2–2½	8	2.0	1–3½
0.5	4	1.8	1–2½	6	2.0	1–2½
0.25	10	0.9	0–1	6	0.8	½–2
0.125	4	1.3	1–1½	..	...	....

4. Tests Based on the Reactions of the Rat or Mouse Uterus

A. DECIDUOMA REACTIONS

The deciduoma reaction in the rat or guinea pig, first reported by Weichert in 1928, depends on the fact that, in these species, the estrogen-primed and progesterone-injected animal is so sensitized that the endometrium responds to injury by the formation of a maternal placental tumor. Results in the earlier literature were qualitative, but Astwood (1939a, b) has investigated the phenomenon in some detail and presents data showing good evidence for the practicability of using it as an assay method. Astwood found that ovariectomized rats primed with estrogen and then treated with progesterone alone or with varying proportions of estrogen responded poorly to uterine trauma. It was found, however, that consistent deciduomata formation occurred when pseudopregnant animals were ovariectomized and then injected with progesterone. This procedure utilizes a natural sensitization of the endometrium, which is only maintained on ovariectomy if exogenous progesterone is supplied.

Adult female rats, 3–5 months old, weighing 150–200 g. are used. Animals showing fully cornified vaginal smears are selected from the colony and subjected to cervical stimulation of the uterus by a faradic current, as described by Greep and Hisaw (1938). Vaginal smears are taken daily thereafter. Four days after application of the cervical stimulus, the rats are ovariectomized, and at the same time the endometrium in one uterine horn is damaged by inserting a needle through the cut tubal end down as far as the cervix. By withdrawing the needle at an angle, the antimesometrial side of the uterine horn is scratched throughout its length.

Injection of progesterone is now started, once daily in 0.1–1.0 ml. of

oil is sufficient, the treatment being continued for 3 days. During the time of treatment, the vagina is frequently examined for signs of external hemorrhage. The animals are killed on the third day from the start of injections, and the traumatized uterine horn examined for gross swelling as compared with the other, untreated horn. It was found that histological examination gives questionable results unless such macroscopic swelling is seen. The degree of swelling was given ratings of from 1–4 plus by comparison with deciduomata produced in intact and uninjected pseudopregnant females by similar traumatization. A rating of 4 was given to uteri showing a reaction equivalent to a 3-day normal deciduoma, a rating of 3 to those equivalent to a 2-day normal deciduoma, of 2 to those equivalent to a 1-day normal response and of 1 to questionable reactions. In no instance in a group of 22 controls was a reaction found, in the absence of injection with progesterone.

With this technic, reactions 3–4 were produced by 0.5–1 mg. of progesterone, 2–3 with 0.25 mg. and 0–1 with 0.1 mg. Astwood's results were not extensive, but suffice to show that the method is workable. External uterine hemorrhage frequently appeared and seemed to be related to the degree of decidual response. It is thought by Astwood to be related to the inability of the injected progesterone to maintain the deciduomata in a fully normal condition, as similar bleeding is shown at the end of pseudopregnancy by rats with deciduomata, and heralds the approach of the next estrous period.

B. STROMAL NUCLEAR HYPERTROPHY

Hooker (1940, 1945) found that the endometrium of the mouse exhibits an apparently specific response to progesterone, consisting in hypertrophy of the stromal nuclei. A dose of about 0.125 mg. of progesterone elicits this reaction when given parenterally, but it was soon found that intra-uterine administration required very small amounts, down to as little as 0.0002 μg.

The instrument used for intra-uterine administration of progesterone is shown in Fig. 8. It resembles the "Agla" micrometer syringe. A ½-ml. tuberculin syringe is mounted on a firm base, and its plunger is controlled by the micrometer bolt. The syringe itself is readily removed for filling and cleaning. Throughout a subsequent study by Hooker and Forbes (1947), the hormone was dissolved in sesame oil. Even as small a volume as 0.01 ml. distends the uterine segment sufficiently to produce frequent obliteration of the tunica propria, hence very small volumes must be used. The standard amount delivered in Hooker and Forbes' work was 0.0006 ml.; more than this amount produced undesirable distention.

The test animal is an adult female mouse, ovariectomized 16 days beforehand and anesthetized with sodium amytal and ether. One uterine horn is delivered through a midventral incision and anchored by a needle passed through the mesometrium (Fig. 9) and a tight ligature is placed near the cranial end of the cornu. The isolation of a segment of uniform length and prevention of leakage is attained by the following technic, as described by Hooker and Forbes (1947):

"A second thread is placed approximately 5 mm. caudal to the first, and a single loose overhand knot is tied. In delivering the horn through the abdominal wall the large vessels that course the mesometrium parallel to the uterus are identified and care is taken to place the anchoring needle and the ligatures between these vessels and the horn. This precaution
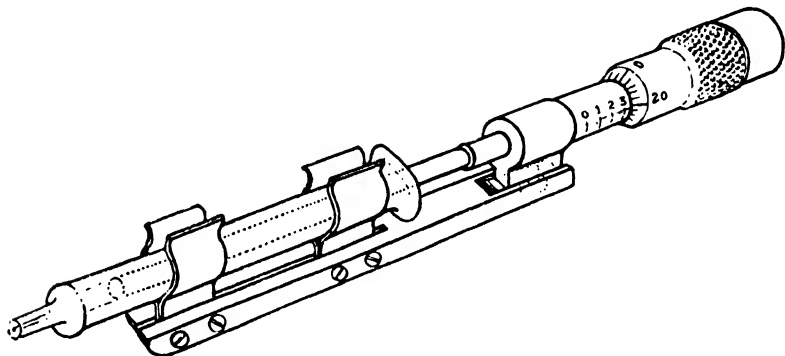
FIG. 8. Apparatus for intra-uterine injections.   (From Hooker and Forbes, 1947.)

preserves the blood supply to the entire uterus. The ends of both ligatures are passed through two slots 5 mm. apart in a device (a comb suffices) clamped horizontally about one and one-half centimeters above the animal. The slots serve to measure a 5 mm. segment of uterus and to draw the threads vertically. The ends of the threads are gripped in artery clamps attached to a horizontal rod. By means of the threads the horn is elevated slightly above the abdominal wall and held securely.

"After making sure that the needle is filled with fluid, the horn is grasped with a forceps about 3 mm. caudal to the posterior-ligature and the needle, bevelled surface up, is inserted into the uterine lumen immediately cranial to the forceps and pushed forward almost to the anterior ligature. The desired volume of fluid is then discharged into the cornu, and the needle is withdrawn. During the withdrawal of the needle tension is placed on the caudal thread sufficient to produce a sharp angle in the horn when the needle is removed. The tension is continuously maintained until the knot is drawn tight. After cutting the threads

close to their knots, the second horn is similarly injected if desired, the organ is returned to the abdominal cavity, and the body wall is closed with silk sutures."

The animal is killed 48 hr. after injection, and the injected uterine segment is fixed in Lavdowsky's fluid (Williams and Hodge, 1943). This produced less shrinkage than other fixatives tested. Paraffin sections 6 μ in thickness were stained with Harris' hematoxylin and eosin.
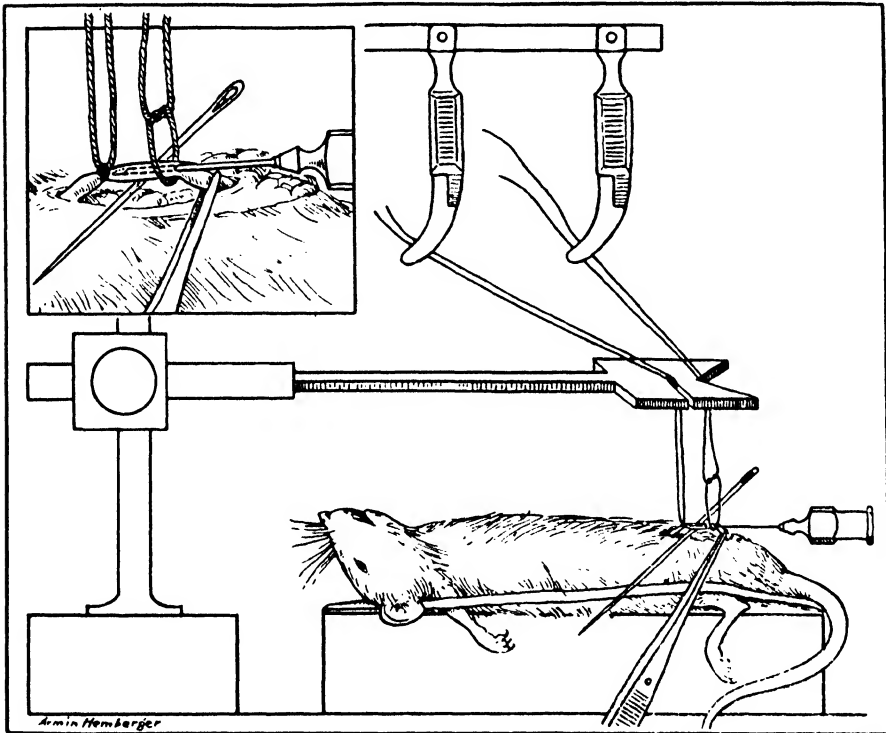
FIG. 9.   Method for intra-uterine injection.   (From Hooker and Forbes, 1947.)

If the interval between injection and autopsy is shortened, the minimal effective dose rises, being doubled at 24 hr. and quadrupled at 6 hr.

The authors' figures illustrating the response are reproduced in Fig. 10, together with typical reactions to other steroid hormones.   Under stimulation from progesterone, the stromal nuclei enlarge and appear smooth, elongated and oval in outline, whereas control sections show shrunken nuclei with clumped chromatin.   In the stimulated nuclei, the chromatin particles are fine and evenly distributed, and there is a conspicuous nucleolus.   All these conditions must be seen before a posi-
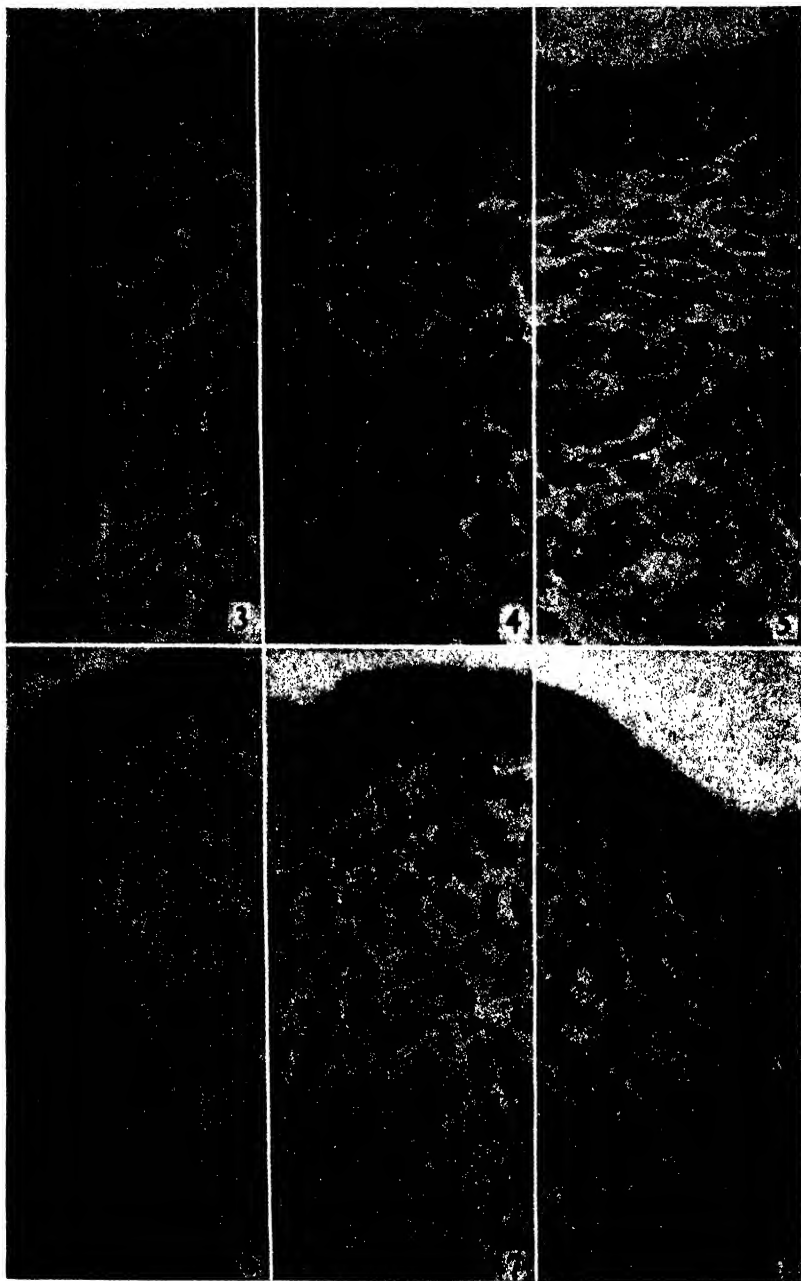
FIG. 10.   For descriptive legend see opposite page.

tive response is scored, and it is necessary to distinguish between the stromal nuclei and the nuclei of the glandular epithelium. The latter do not appear to be affected by progesterone and can be recognized as belonging to uterine glands, if necessary, by examining adjacent serial sections. They may appear to have no cytoplasm around them and are irregularly rounded with inconspicuous nucleoli and comparatively coarse chromatin particles which are frequently clumped together near the nuclear membrane. The stromal nuclei are most readily identified in the antimesometrial portion of the endometrium, which is thicker than on the opposite side. Under the distention produced even by 0.0006 ml. of oil, this side may be stretched sufficiently to make identification of the stromal nuclei difficult.

The *minimal effective dose* was defined by Hooker and Forbes as the least amount which induces a positive response in any stromal nuclei and is said to be consistently 0.0002 μg. of progesterone. When more than 0.005 μg. of progesterone was given, responses were seen in untreated, contralateral horns. This method of scoring the response could clearly be adapted in a quantal assay, in which it would seem that 100% of positive responses could be expected with the minimal effective dose as described, and the median effective dose would be a quantity less than this. No data are presented by Hooker and Forbes which permit an estimate of this dose, or of the slope of the dose-response line, and thus the accuracy of the method is unknown.

Specificity was examined in some detail. Tobin (1941) has reported that sesame oil is not always inert, but the injection of this oil alone did not produce characteristic reactions. On systemic administration, desoxycorticosterone has progestational activity, while testosterone is a weaker progestogen (Selye and Masson, 1943). After the injection of up to 6 μg. of desoxycorticosterone or of up to 0.48 μg. of testosterone acetate into the uterus, no reactions similar to those produced by pro-

Fig. 10. Sections of endometria of mice ovariectomized 16 days previously. (From Hooker and Forbes, 1947.)

3. No treatment. The stromal nuclei are shrunken and have clumped chromatin.
4. After intra-uterine injection of 0.00075 ug. of progesterone. A characteristic stromal nucleus is oval and has a conspicuous nucleolus and fine, evenly dispersed chromatin particles.
5. After sesame oil alone. The stromal nuclei are spindle-shaped.
6. After 6.0 μg. of desoxycorticosterone acetate. Edema is conspicuous; the stromal nuclei show little change. Part of a gland appears at the left.
7. After 0.48 μg. of testosterone. In being smaller and wrinkled, the stromal nuclei fail to meet the criteria for a positive response.
8. After 0.0075 μg. of estradiol. The chief response is heightening of the epithelium. The stromal nuclei are somewhat enlarged.

gesterone were seen; although the nuclei were irregularly swollen, they had coarse chromatic granules (Fig. 10) and did not resemble those exhibiting a positive response as defined above. Up to 0.00075 μg. of estradiol or 0.6 μg. of estrone also failed to elicit positive responses, and the simultaneous administration of estrogens and progesterone did not alter the response to progesterone.

This test was then used by its authors in examining the action of impure extracts. A crude extract of sows' corpora lutea gave the same value for progestogen content when assayed by the intra-uterine technic in the mouse and by the Allen (1930) method. However, only 4 rabbits were used, and the comparison must have been very approximate. Citrated plasma from 2 rabbits bled on the sixth day of pseudopregnancy assayed at the equivalent of 4.3 μg. of progesterone per milliliter, whereas the content of similar plasma from ovariectomized rabbits were nil. Serum from ovariectomized mice also gave no response, whereas serum from similar mice injected 6–7 hr. previously with 2 mg. of progesterone (subcutaneously in oil) showed a content of between 0.33 μg. and 8.25 μg. of progesterone per milliliter. Liquor folliculi from those sows' ovaries furnishing the corpora lutea referred to above also showed a content of 4.13–8.25 μg./ml., but in the absence of data obtained with liquor folliculi from ovaries *not* also possessing corpora lutea it would seem uncertain whether the progestogen detected originated in the preovulatory follicles from which the fluid was drawn, or whether it had diffused from the adjacent luteal tissue.

5. Further Test Methods

A. PREGNANCY RESPONSE IN THE FELINE UTERUS

Van Dyke and Chen (1939) used the response of the cat's uterus to sympathetic stimulation as a tentative assay method for progesterone. Dale (1906) and Cushny (1906) first described the reversal of the effect of sympathetic stimulation on the uterus of the pregnant cat, which contracts instead of relaxing as it does in the non-gravid animal. Van Dyke and Gustavson (1929) showed that this could be demonstrated in the ovariectomized non-pregnant animal which had received injections of crude corpus luteum extract. The response to progesterone itself was then investigated by Kennard (1937) and Van Dyke (1937). The latter author pointed out the possibility of assaying progesterone-containing extracts by inducing a pregnancy-response in normal or ovariectomized cats, which was then more fully investigated in collaboration with Chen.

The method is rather tedious and seems unlikely to be developed

further. It used high doses of progesterone (0.25–0.75 mg.), takes a minimum of a week to perform, and would be very difficult to run with many animals simultaneously. It is included in the present survey mainly because no method of assaying progesterone is very easy to handle, and it may be of interest in particular circumstances.
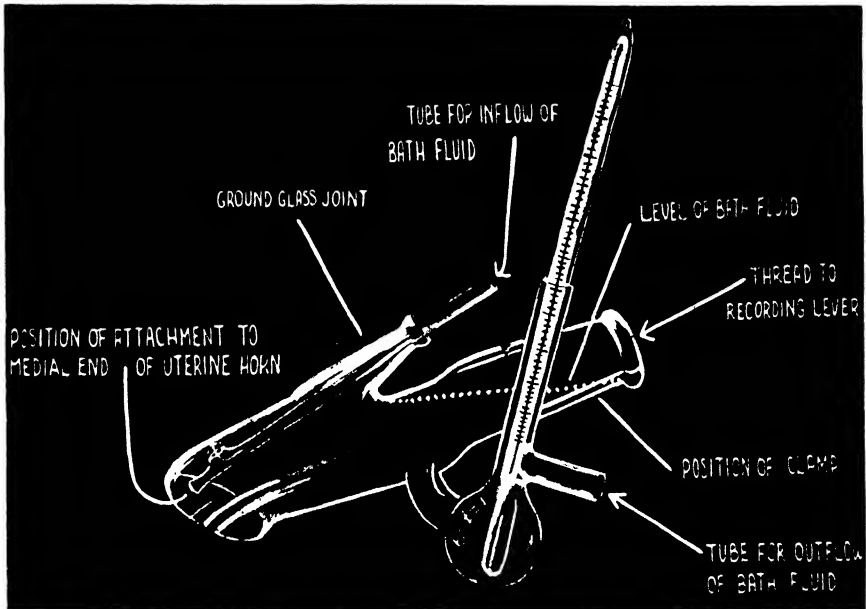
Fig. 11. Glass apparatus for recording uterine contractions in situ. (From Van Dyke and Chen, 1939.)

Either intact or ovariectomized cats are used, and subjected to the following schedule of operations:

Day of test	Procedure
1	500 I.U. of estradiol benzoate (subcut?)
2	500 I.U. of estradiol benzoate (subcut?)
3	No treatment
4	One-third total dose of progesterone
5	One-third total dose of progesterone
6	One-third total dose of progesterone
7	Determination of response to adrenalin (i.v.) and hypogastric stimulation

The uterine response is determined under anesthesia, the uterus being exposed by a low lateral abdominal incision. The medial part of the left uterine horn is tied by a string leading to an isotonic lever, and the uterus is inserted into a glass apparatus shown in Fig. 11. This is, in effect, a

bath for keeping the uterus moist and at 37°C. *in situ*, and the incised abdominal wall is tightly clamped around the lower part of it to retain the bath fluid, which is of the following composition:

Cations			Anions	
Na	144.0 mm./l.	Cl		122.0 mm./l.
K	6.2 mm./l.	HCO$_3$		30.0 mm./l.
Mg	1.0 mm./l.	H$_2$PO$_4$ }		1.0 mm./l.
Ca	0.75 mm./l.	HPO$_4$ }		
pH	7.4			

The response to 1–20 μg. of intravenous adrenalin and to hypogastric stimulation is tested and recorded on a kymograph. It is scored merely as positive (contraction) or negative (relaxation or no effect). Dose-response data for progesterone are shown in Table II. Up to 30 mg. of

TABLE II

Dose-Response Data for the Assay of Progesterone by the Van Dyke Method. (Adapted from Van Dyke and Chen, 1939)

No. of cats	Ovariectomized	Dose of progesterone in mg.	Per cent positive response
21	No	0.25	19
22	No	0.50	65
21	No	0.75	81
5	No	1.00	80
20	Yes	0.25	15
22	Yes	0.50	59
20	Yes	0.75	70
10	Yes	1.00	70

pregnanediol and 38 mg. of testosterone propionate did not cause a typical pregnancy response; relaxation mostly occurred, sometimes preceded by a slight, transient contraction. However, even progesterone produced diphasic responses in some animals and sometimes the results with adrenalin and hypogastric stimulation differed, so it would appear that in a proportion of cases the test is difficult to interpret.

B. SEXUAL RECEPTIVITY, NIDATION OF OVA, AND OTHER TESTS

Various other suggested methods have been based on the induction of sexual receptivity in the estrogen-primed guinea pig, which is not produced by estrogen alone (Hertz *et al.*, 1937) and the nidation of eggs in the spayed rabbit (Courrier, 1935). These methods have received no critical attention, and in view of the much greater sensitivity of the test developed by Hooker and Forbes, seem unlikely to attract further attention for biological assay.

In common with other steroids, progesterone causes a lengthening of the ovipositor in the bitterling (de Wit, 1938a, b, c; 1940). It has

been suggested as a test method, because progesterone is much more potent than androgens, which, in common with it, act measurably after a latent period of 1 hr., and because estrogens do not take measurable effect until after a latent period of about 10 hr.

II. RELAXIN

1. Introduction

Relaxation of the symphysis pubis occurs in late pregnancy in many species and has been especially studied in rodents. Hisaw (1926) first demonstrated that such relaxation is under hormonal control and called the responsible substance relaxin. Hisaw's studies were made with guinea pigs, in which relaxation of the symphysis pubis may also be produced by estrogen and progesterone (de Fremery et al., 1931; Courrier, 1931; Tapfer and Haslhofer, 1935; Haterius and Fugo, 1939); the existence of a separate non-steroid hormone was therefore doubted by many investigators. The action of relaxin is also dependent on previous priming with estrogen, and it is suggested by Hall and Newton (1947) that it may no more than a potentiator of the action of estrogen in causing relaxation. There is, however, no practical doubt that relaxin exists as a separate entity with the property of causing separation of the two halves of the pubic symphysis in the estrogen-treated mouse or guinea pig. Abramowitz et al. (1944) showed that 500 times the amount of relaxin necessary to produce a positive response in the guinea pig was without either estrogenic or progestational effect and Hisaw et al. (1944) further succeeded in differentiating the actions of relaxin and progesterone and produced evidence that the latter acts only by stimulating the production of relaxin itself. The relaxation produced by progesterone is delayed in comparison with that produced by relaxin, and does not occur in the castrated and hysterectomized animal, in which relaxin still acts. Relaxin was also detected in the blood of progesterone-treated female rabbits but not in the blood of similarly treated castrated males or castrated and hysterectomized females.

Estrogen in large doses causes relaxation, but the dose required to prime animals for the action of relaxin is small and without effect alone. As mentioned above, it remains a definite possibility that relaxin acts as a potentiator of estrogen, since it is ineffective in the absence of prior estrogen treatment, but if so, its action in so potentiating estrogen must be powerful.

2. Sources of Relaxin

Relaxin was first discovered in the serum of pregnant rabbits (Hisaw, 1926), and afterwards in several other pregnant mammals (Hisaw, 1929;

Pommerenke, 1934; Abramson *et al.*, 1937). It is also found in extracts of rabbit placenta and of sows' corpora lutea (Hisaw, 1926, 1927, 1929). This has been confirmed by many subsequent investigators.

Methods of preparation are described by Fevold *et al.* (1930), Abramowitz *et al.* (1942), Abramowitz *et al.* (1944) and Albert *et al.* (1946). The hormone has not been obtained in a pure state, but the amorphous material so far obtained, with an activity of about 30 guinea pig units per milligram, is slightly soluble in water and 95% alcohol, insoluble in common organic solvents, with an isoelectric point of 5.4–5.5. Its activity is destroyed by alkalis, oxidizing agents, and proteases (Abramowitz *et al.*, 1944). Solutions in acid are stable. It is suggested that the hormone may be a peptide.

3. Methods of Assay

A. THE GUINEA PIG TEST

Fevold *et al.* (1930) defined as a unit that amount of relaxin which is required to cause a definite loosening of the pelvic ligaments within 10 to 12 hr. of a single injection. Serial dilution were used until a negative response was obtained. Other investigators, always using only a few animals per test, were content to report the presence or absence of relaxin in material tested, until Abramowitz *et al.* (1944) put the method onto a more quantitative basis.

These authors prepared a large stock of relaxin to serve as a standard and investigated many of the variables of the test. Defatted luteal tissue from sow ovaries were extracted with 2% HCl and inert material removed by the addition of NaCl to 1 molar concentration followed by neutralization. The hormone was precipitated with saturated $(NH_4)_2\text{-}SO_4$. One milligram of this preparation was about equivalent to 1 guinea pig unit as described below.

Within the range 350–800 g., the weight of the animal was found to have little influence on response. Seasonal variation was also negligible. Estrogen pretreatment was found to be essential, but the effects were found to persist for over 7 days following the last injection of estrogen. Constant injection with estrogen resulted in greater sensitivity to relaxin as time went on, but the percentage of spontaneous relaxations increased, and it was therefore decided not to attempt the use of permanently treated animals. A dose of 0.73 µg. of estradiol for 4 days was found to be suitable for assays, followed on the fifth day by the injection of relaxin. The maximum response was found at 6 hr. after injection, with a definite decrease by 12 hr. The recommended test method is thus as follows.

Groups of (at least) 10 to 12 virgin guinea pigs are taken of a body weight of at least 250 g., ovariectomized and rested until they have attained a minimum body weight of 350 g. and a maximum body weight of 800 g. Animals of more than 800 g. are rejected more because fat accumulates around the pelvis and renders palpation of the symphysis pubis difficult than for any other reason. All acceptable animals are then injected with 0.83 μg. of estradiol in oil daily for 4 days, and on the fifth day each receives a single dose of relaxin subcutaneously in 1 ml. of distilled water. Six hours later, the extent of pelvic relaxation is
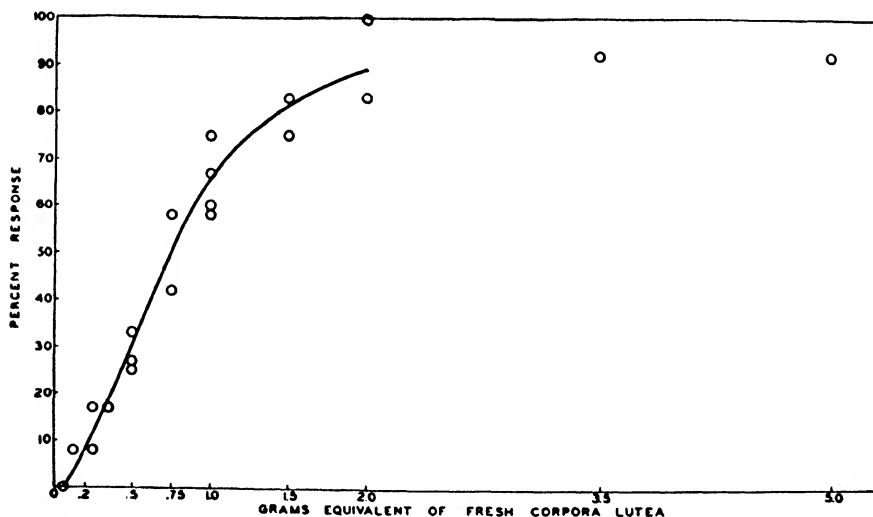
Fig. 12. Percentage of guinea pigs showing relaxation of the symphysis pubis after single injections of relaxin with 12 animals per group. (From Abramowitz et al., 1944.)

judged subjectively by palpation, and those showing an unmistakable relaxation are scored as positive. The results obtained by the authors of the technic are shown in Figs. 12 and 13, each point representing 12 animals. It is clear that a linear dose-response relationship would hold over a sufficient range for assay by the standard probit methods, although the authors did not employ probits. The slope of the probit-log dose line is about 3.0, hence the expected minimal limits of error with 20 animals per group in a 4-point assay (80 animals all told) would be about 60–160%, less if a standard slope could be used, determined with little error.

A guinea pig unit of relaxin was defined as that amount of hormone inducing an unmistakable relaxation in about two-thirds of a group of

12 guinea pigs treated as above.   It would be far preferable, of course, to define, say 1 mg. of the provisional standard as prepared by Abramowitz and his colleagues as the unit and to assay all other preparations in terms thereof.

The same animals can be used repeatedly, with a rest period of a week between consecutive assays.   Attempts to shorten this period or to keep animals in a state of permanent sensitivity to relaxin resulted in spontaneous relaxation in the test animals as mentioned earlier.
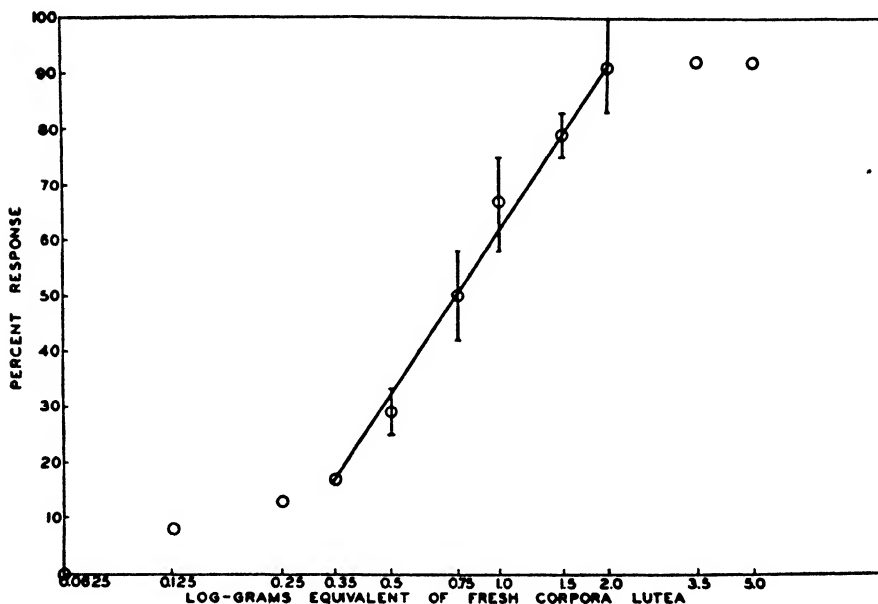
FIG. 13.   The data in Fig. 12 plotted on a semilog scale.   The vertical bars enclose the extreme responses in groups of 12 animals.   (From Abramowitz et al., 1944.)

B. THE MOUSE TEST

Hall and Newton (1946a, b) showed that separation of the pubic bones of the mouse can be demonstrated in x-ray photographs from the thirteenth day of pregnancy onwards, and that this is also produced by relaxin as in the guinea pig.   The advantage of photography in the mouse is that the response may be measured in terms of millimeters separation instead of having to be scored quantally.   Estrone alone also produces this separation; relaxin alone has only a slight effect, again as in the guinea pig.   Progesterone in the mouse, however, does not modify the response to estrone or to estrone plus relaxin, in doses of up to 1 mg. per day (Hall and Newton, 1947).

Hall and Newton's earlier work employed the simultaneous administration of estrone and relaxin, after 2 days of priming with estrone alone. They subsequently found, however, that a method similar to that used by Abramowitz *et al.* (1944) is workable and preferable for assays. Priming periods of from 2 to 16 daily injections of 1.5 μg. of estrone were tried, using ovariectomized virgin adult female mice of the Parkes' albino strain. A single injection of 0.2 ml. of relaxin extract prepared from the serum of pregnant rabbits was given 24 hr. after the last estrone injection and the x-ray photographs were taken 6 hr. and 24 hr. later, and compared with similar photographs taken immediately before giving relaxin. Some results of this investigation are shown in Table III.

TABLE III

Effect on the Symphysis Pubis of Previously Untreated, Virgin, Spayed Mice of a Single Injection of 0.2 ml. of Relaxin Extract Given 24 hr. after Varying Numbers of Daily Injections of 1.5 μg. of Estrone (From Hall and Newton, 1948)

(i) No. of group and (in brackets) No. of exp.	(ii) No. of daily injections of estrone	(iii) Serial No. of relaxin extract used	(iv) No. of mice used	(v) Average width of interpubic gap immediately before administration of relaxin (mm.)	(vi) Average width of interpubic gap 24 hr. after administration of relaxin (mm.)	Average increase in width of gap during last 24 hr. (mm.)	
						In each experiment	In each group
I	2	26	5	0.21	0.52	0.31	0.31
II [i]	4	26	4	0.26	0.95	0.69	0.59
[ii]		43	7	0.19	0.73	0.54	
III [i]	6	26	5	0.47	1.26	0.79	0.73
[ii]		43	10	0.29	0.99	0.70	
IV	7	27	7	0.23	1.37	1.14	1.14
V [i]	8	26	5	0.41	1.88	1.47	1.40
[ii]		39	5	0.10	1.50	1.40	
[iii]		43	10	0.39	1.75	1.36	
VI [i]	9	24	5	0.34	1.86	1.52	1.36
[ii]		28	6	0.44	2.47	2.03	
[iii]		30	10	0.23	1.11	0.88	

It was found that in previously untreated, spayed virgin mice, estrone alone in daily doses of 1.5 μg. produces slight interpubic separation of not more than 0.5 mm. After relaxin, little effect is seen at 6 hr., but at 24 hr. the interpubic gap widens by up to 2 mm. The effect of relaxin given in a single injection is increased with increasing periods of pre-

treatment with estrogen up to 8 or 9 days, but probably not thereafter. The investigation was not carried as far as establishing a dose-response curve with graded doses of relaxin, and it is therefore impossible to recommend a specific assay procedure, or to estimate its probable limits of error. A statistical appendix to Hall and Newton's (1948) paper, by R. L. Plackett, gives details of the variation within groups on the same treatment, from which it appears that the standard deviation is correlated with response.

As far as they have taken matters, the authors incline to recommend the following procedure for test purposes.

Sexually mature albino mice are ovariectomized and subsequently primed for between 5 and 8 days with 1.5 μg. of subcutaneous estrone dissolved in 0.05 ml. of ground-nut oil. A constant period of injection is, of course, used in any one test. Twenty-four hours after the last estrone injection, the pelvis is examined by x-rays, and the animal receives 0.2 ml. of relaxin extract simultaneously. X-ray examination follows again 24 hr. later, and the increase in pubic separation measured. These measurements are taken with a dental x-ray machine. The x-rays are directed at 45° to the horizontal in the animal's sagittal plane onto its perineum, while the animal is in the normal position. The photographic film is placed on the bench beneath the pelvis, and the pubic region is pressed down onto it while photography takes place, the tail is also held over the animal's back. A lead screen is used to protect the hands of the operator. An exposure of 0.85 sec. with the anticathode 19–20 cm. from the perineum of the mouse was found to be optimal. The recorded gap is the shortest distance between the bones, and although the shadow of the pelvis as a whole is distorted, it was found that the method gives a satisfactory estimate of the interpubic gap.

When the pubic bones have once separated, a considerable reaction may be produced by estrone alone and it would not seem feasible to use a mouse more than once in this test.

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Chemical Assay of Estrogens and Pregnanediol

By G. A. D. HASLEWOOD

CONTENTS

I. Introduction

In this article, an attempt has been made to provide the following information:

1. An account, not necessarily fully documented, of methods used for the chemical assay of the estrogens and of pregnanediol.

2. Full experimental details of methods used for the following:

    a. Hydrolysis of urine for estrogen estimation.

    b. Extraction and separation of the chief natural estrogens from urine.

    c. Kober colorimetric estimation of the natural estrogens from urine.

    d. Girard separation of weakly phenolic ketones from non-ketones from urine.

    e. Venning gravimetric estimation of urinary sodium pregnanediol glucuronidate.

    f. Accurate estimation of urinary pregnanediol.

    g. Shorter estimation of urinary pregnanediol.

    h. Naphthoresorcinol reaction as applied to the estimation of pregnanediol glucuronide.

The methods given in full have been selected as far as possible on the basis that they represent the most fully developed and most rigorously tested procedures available at the present time.

3. An indication of results obtained by use of the methods reviewed, together with a critical summary of the possible value of the methods.

II. Estrogens

Three natural estrogenic hormones, estrone, estradiol, and estriol, have been shown to be present in human pregnancy urine, whereas at least six similar compounds can be isolated from the urine of pregnant mares. Estrone and α-estradiol have been obtained from ovarian tissue, whereas estrone, α-estradiol, and estriol have been isolated from human placenta.

Part at least of the estrogen in the blood of pregnant women and of pregnant mares is in the conjugated water-soluble form (Mühlbock, 1937, 1939; Rakoff *et al.*, 1943), and the same may be true of human placental estrogen (Collip *et al.*, 1934). The occurrence of conjugated estrogens in urine is discussed below.

The three most frequently occurring natural estrogenic hormones contain the phenolic grouping
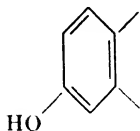
which seems to be important for estrogenic activity. The chemical methods of assay likewise depend on the presence of the phenolic grouping, although the ketonic properties of estrone and the more strongly acidic nature of estriol (as compared with estrone and estradiol) have proved valuable as a means of separating these substances for analysis.

For all practical purposes, the value of the chemical assay of estrogens is limited at present to human urine of pregnancy and, in some instances, as a guide in working up concentrations of natural estrogens, e.g., Schachter and Marrian, 1938; other claims which have been made are mentioned below, but full experimental detail is here concerned with human pregnancy urine alone.

1. Chemical Assay of Estrogens in Urine

A. HYDROLYSIS AND EXTRACTION OF ESTROGENS

It has been shown that the greater part, if not all, of the estrin in human and equine pregnancy urine is in the conjugated form (see, e.g., Cohen et al., 1935; Cohen et al., 1936; Schachter and Marrian, 1936, 1938). Cohen and Marrian (1936) isolated estriol monoglucuronide from human pregnancy urine and Schachter and Marrian (1938) obtained estrone sulfate from pregnant mares' urine. Butenandt and Hofstetter (1939) have provided evidence that in human urine of pregnancy, also, estrone sulfuric acid ester probably occurs.

Venning et al. (1937) proposed that the urine should first be extracted with n-butanol, a procedure which they stated would extract "both free and combined forms of estrin"; however the butanol extraction method has not found favor with most other workers.

Cohen and Marrian (1934, 1935) heated pregnancy urine, adjusted to approx. pH 1.0 with hydrochloric acid, in an autoclave at 120°C. and 15-lb. pressure for 2 hr. The estrogens were then extracted from the cooled urine with ether. Smith and Smith (1935) and Smith et al. (1939) hydrolyzed the combined estrogens by making the urine approx. 1.5 N by the addition of concentrated hydrochloric acid, and then boiling for 10 min. under a reflux condenser. The hydrolyzed material was also extracted with ether.

Gallagher *et al.* (1935) described an apparatus for continuous extraction of estrogens with benzene from acid-hydrolyzed urine.

Leiboff and Tamis (1938) extracted estrogen from acid-hydrolyzed urine with chloroform in a continuous-extraction apparatus.

Edson and Heard (1939) hydrolyzed the "biologically impotent water-soluble conjugated estrogens" of pregnant mares' urine by acidifying the urine to *p*H 0.4–0.6 and allowing it to stand at room temperature for at least 4 weeks.

Dingemanse *et al.* (1939) extracted the urine during acid hydrolysis by adding a layer of benzene. More recently, Stevenson and Marrian (1947), although accepting the Smith and Smith method of hydrolysis, modified it slightly by increasing the time of heating (see below).

Smith and Smith (1937) and Smith *et al.* (1939) showed that the yield of estrogen (as determined by biological test) could be much increased by the addition of zinc dust during the hydrolysis. This phenomenon was further examined by Smith and Smith (1941) who found that estrone alone, in pure solution, was affected by the process, being converted, at least partially, to a more potent product: the potency of pure estradiol and estriol was not altered. Smith and Smith could not account, on chemical grounds, for the whole of the increased potency, but concluded, "Zn-HCl hydrolysis is not suitable for the study of urinary estrogens as such."

Methods of hydrolysis of conjugated estrogens in pregnancy urine were compared by Van Bruggen (1948) using bioassay. He concluded *inter alia* (1) that 10-min. hydrolysis with 15 vol. % conc. HCl did not hydrolyze all the conjugated estrogens present; (2) that pressure hydrolysis at 120°C. and *p*H 1.0 could be recommended for a routine procedure; (3) that the usual 4 ether extractions might not remove all "supposedly ether-soluble forms"; (4) that the use of butanol as a solvent was of definite promise (see, however, above). Van Bruggen further found that increased time of heating in the Smith and Smith hydrolysis did, in fact, give increased ether-soluble estrogens.

In the light of these and other findings, it would appear that either pressure hydrolysis, or the slightly modified boiling hydrolysis of Smith and Smith constitute the most reliable known methods of liberating estrogen from its conjugates in urine.

B. HYDROLYSIS OF CONJUGATED ESTROGENS IN URINE (STEP A)

i. *Pressure Hydrolysis of Cohen and Marrian (1935)*. The sample of 100 ml. of urine is adjusted to *p*H 1.0 with HCl. It is then further treated with 3.3 ml. of 12*N* HCl (4.0 ml. of 10*N* HCl) and heated in an autoclave at 120°C. and *ca.* 15-lb. pressure for 2 hr.

ii. *Open-Flask Acid Hydrolysis:* (Smith and Smith, 1935; Stevenson and Marrian, 1947). Twenty-four hour specimens, collected with toluene as preservative, are diluted to a volume of 2.5 l. before removing samples for hydrolysis. One hundred milliliters of the diluted urine is heated to boiling under reflux, and, after the addition of 15 ml. of $10N$ HCl down the condenser, boiling is continued for 30 min.

Estrogens may be extracted with ether (see above), and, less effectively, benzene. Bachman and Pettit (1941) have shown that benzene can be used to extract estriol (for which purpose it is otherwise inefficient) provided that the hydrolyzed urine is previously saturated with sodium chloride. Talbot *et al.* (1940) state that carbon tetrachloride may be used in a suitable continuous extraction apparatus. The solvent used for extraction of estrogens may be washed with sodium carbonate or bicarbonate to remove stronger acids. Further purification can be effected as described below.

C. EXTRACTION OF TOTAL ESTROGENS FROM ACID-HYDROLYZED URINE (STEP B)

i. (*Stevenson and Marrian, 1947.*) The 100-ml. acid-hydrolyzed sample of urine is cooled and extracted once with 100-ml. and twice with 50-ml. samples of purified ethyl ether.* The combined ethereal extracts are washed 3 times with 25-ml. portions of 5% (w/v) sodium bicarbonate and the combined washings back-extracted once with 20 ml. of ether. The washed ethereal extract is combined with the back-extract and evaporated to dryness. The residue is warmed with *ca.* 3 ml. of ethanol and 100 ml. of benzene added. This benzene solution is then extracted once with 50-ml. and twice with 25-ml. portions of N NaOH. The combined NaOH extracts are acidified with 15 ml. of $10N$ HCl and extracted once with 100-ml. and twice with 50-ml. portions of ether. The combined ethereal extract is washed twice with 20-ml. portions of 5% (w/v) NaHCO₃ and the washings back-extracted once with 20 ml. of ether. The combined ethereal extract is washed three times with 20-ml. portions of water and evaporated to dryness. The residue consists of the total ether-soluble phenolic fraction of hydrolyzed urine.

ii. (*Van Bruggen, 1948.*) The 100-ml. sample of acid-hydrolyzed urine is cooled and saturated with NaCl. The estrogens are extracted from the mixture with four 50-ml. portions of purified ethyl ether. The combined ether extracts are washed with 9% (w/v) NaHCO₃, dilute HCl and distilled water. The ether is removed by distillation.

* "Peroxide-free" ether can be prepared by vigorously shaking ether of good quality with a little freshly made ferrous sulfate solution (1 g./100 ml.) decanting the ether and distilling.

By the means described above, the urine may be hydrolyzed and the total ether-soluble phenolic material, which contains the estrogens, may be obtained. Further fractionation of this material may be effected into (1) stronger (more acidic) phenols (estriol), (2) ketonic phenols (estrone), and (3) weaker phenols (estrone and estradiol).

Cohen and Marrian (1935) separated the "strong" and "weak" phenolic fractions by washing the ethereal extract from hydrolyzed urine with dilute sodium carbonate to eliminate acids, extracting the ether with 0.1 N sodium hydroxide to remove estriol, back-washing the sodium hydroxide with ether, evaporating the residual ether and back-washings to dryness, taking the residue up in toluene and extracting this with N sodium hydroxide to remove estrone. Although Cohen and Marrian did not claim that the separation was quantitative, Smith, Smith, and Schiller (1939) stated that this method was "very satisfactory for all practical purposes."

Mather (1940, 1942) and Bachman and Pettit (1941) showed that estriol can be extracted from benzene by aqueous sodium carbonate, whereas estrone and estradiol remain in the benzene. In the latter fraction (estrone and estradiol), estrone can be determined by the Zimmerman reaction.

D. SEPARATION OF ESTRIOL FROM ESTRONE AND ESTRADIOL IN URINE EXTRACTS (STEP C)

(*Bachman and Pettit, 1941*)—extraction of urine. One hundred milliliters of freshly hydrolyzed urine was cooled and diluted with an equal volume of distilled water. Fifty-six grams of sodium chloride was added. The resultant solution was extracted four times with an equal volume of benzene. The combined benzene extracts were washed with 0.02 volume of a freshly prepared solution of sodium bicarbonate (9 g. of anhydrous $NaHCO_3$/100 ml.) and concentrated to about 35 ml.

In an alternative procedure the diluted hydrolyzate, without the addition of salt, was extracted with 1 equal volume and 2 half volumes of ether. The combined ether extracts were washed with 0.02 volume of the above sodium bicarbonate solution and taken to dryness. The residue was dissolved in a minimal volume of ethanol (less than 0.5 ml.), and the solution was diluted with 35 ml. of benzene.

Fraction T (containing estriol). The benzene extract (35 ml.) was extracted once with 1.0 and twice with 0.5 volume of sodium carbonate solution (9 g. of anhydrous Na_2CO_3/100 ml.) and once with 0.1 volume of water. The purification of the benzene phase will be described below. The aqueous Na_2CO_3 extracts were combined, acidified to a pH of less

than 6 with hydrochloric acid, and then extracted three times with 0.5 volume of ethyl ether.   The combined ether extracts were washed twice with 0.1 volume of the above sodium bicarbonate solution and taken to dryness.   The residue was dissolved in 0.5 ml. or less of ethanol; the solution was diluted with 50 ml. of benzene and washed with 1 ml. of the sodium bicarbonate solution.   The benzene phase was transferred to a clean separatory funnel and extracted with three equal volumes of water. The aqueous phase was evaporated to dryness *in vacuo*.   The residue was dissolved in a measured volume of ethanol, from which aliquots were transferred to colorimeter tubes and evaporated to dryness for testing.

Fraction OD (containing estrone and estradiol).   The benzene extract (35 ml.) after the removal of fraction T, was washed once with 0.25 volume of diluted sulfuric acid (4 parts by volume of the concentrated acid to 5 parts of water) and twice with 0.5 volume of water.   It was then extracted 4 times with an equal volume of N sodium hydroxide.   The alkaline extracts were combined, acidified to a pH of 6 or less with hydrochloric acid, and then extracted once with 1.5 and twice with 0.75 volume of benzene.   The combined benzene extracts were concentrated to about 50 ml. and were then washed successively with 0.25 volume of diluted sulfuric acid (4:5), twice with 0.5 volume of sodium carbonate solution (9 g. Na_2CO_3/100 ml.), and twice with 0.5 volume of water.   The benzene was evaporated to dryness, and the residue was taken up in a measured volume of ethanol, from which aliquots were transferred to colorimeter tubes for evaporation and test.

Estrone can be removed from the total urinary extracts by means of a reagent reacting with ketones.   Thus, e.g., Smith *et al.* (1939) used semicarbazide treatment to effect the removal of estrone, then estimated by difference.   More recently, Talbot *et al.* (1940) separated the estrone with Girard's "Reactif T"; however, Bender and Wilson (1947) report that this method includes a large proportion of "ketonic phenols" which are definitely not estrone.   In the present state of knowledge, it may be doubted whether there exists a satisfactory method of separating estrone quantitatively and in a pure state from urine extracts.

Since, however the Girard separation is frequently used in fractionation of urinary and other extracts (see, for example, the useful review by Pincus, 1945), and therefore, it might be of value to many workers, a brief account of it is given here.   In general it may be said that (1) a large excess of pure *dry* Girard's reagent T over the ketonic material thought to be present should be used, and (2) the extracts treated; the apparatus and solvents (ethanol and acetic acid) employed in making the Girard complex must all be as *dry* as possible.

E. GIRARD SEPARATION OF WEAKLY PHENOLIC KETONES

(E.g. *Talbot et al., 1940; Bender and Wilson, 1947.*) The residue to be separated is dissolved in 4 ml. ethanol, together with 0.5 ml. of pure acetic acid and 0.5 g. of Girard's reagent T. The mixture is gently boiled under reflux for 0.5 hr. with a $CaCl_2$ seal on the condenser. The cooled mixture is carefully treated with 40 ml. of ice water and 3 ml. of chilled 2.5N NaOH. The *non-ketonic fraction* is extracted with ether (4 $\times$ 40 ml.) and the ether is washed with 2 $\times$ 25 ml. of 0.1N NaOH and 2 $\times$ 25 ml. of water. The aqueous portion containing the ketones is acidified with 1 ml. of conc. H_2SO_4, and after addition of 60 ml. of water and 40 ml. of ether is left for 2 hr. at room temperature. *Ketones* are then extracted with ether (4 $\times$ 25 ml.), and the ether extract is washed with 25 ml. of 0.1N NaOH and 3 $\times$ 25 ml. of water. Both ethereal extracts are dried with Na_2SO_4 and evaporated to dryness.

The final chemical estimation of the phenolic substances may be done in a variety of ways; the only method which has been sufficiently tested to be reported here fully is that originally due to Kober (1931). Kober showed that if estrone or estriol are heated with a mixture of phenol and concentrated sulfuric acid, and the mixture is then diluted with water, a clear red or pink color is produced which is apparently highly specific for phenols of the natural estrogen type. Cohen and Marrian (1934) investigated the reaction from a quantitative point of view and showed that reproducible results could be obtained with known amounts of estrone and estriol, although a linear relationship between color and concentration did not appear to exist. They found that with extracts of urine a persistent brown color seriously interfered with the final colorimetric estimation. The history of attempts to correct for this effect has been recently given by Marrian (1948a) and Stevenson and Marrian (1947) as follows.

"Urinary estrogen-concentrates usually contain substances which yield a brown colour in the Kober reaction. This brown colour shows considerable light absorption at 520 mμ, so that its presence in the final Kober reaction mixture results in an over-estimation of the estrogen actually present. With human urines collected during mid- and late pregnancy and containing more than about 10 mg. estrogen/24 hr., the ratio of estrogen pink to non-estrogen brown colour is so high that the error is negligibly small. With urines containing less estrogen, however, the over-estimation caused by this brown colour may become considerable.

"The attempts that have been made to minimize this brown colour error can be classified under two main headings: (i) Attempts to measure the brown colour and then to correct for it. (ii) Attempts to eliminate without loss of estrogen the substances that give rise to the brown colour.

"Cohen and Marrian (1934) attempted to correct for the brown colour by measuring the pink in the reaction mixture before and after treatment with hydrogen peroxide—a treatment which as shown by Kober (1931) rapidly discharges the estrogen pink colour. This method was unsatisfactory since some fading of the non-estrogen brown colour resulted from the peroxide treatment.

"A better method was devised by Venning et al. (1937). They showed that the brown colour absorbs strongly at 420 mμ while the estrogen pink is nearly transparent at this wave-length. Accordingly, by measuring the absorption at 420 mμ and by assuming that the spectrophotometric characteristic of the brown colour was constant from urine to urine, they were able to calculate how much of the observed absorption at 520 mμ was due to the brown colour. According to Bachman and Pettit (1941), however, it cannot be safely assumed that the brown colours from different human urine specimens are spectrophotometrically identical.

"Jayle et al. (1943) made use of the instability of the estrogen pink colour in the presence of acetone for a method of brown colour correction. They used the β-naphthol reagent, and the reaction was carried out in the presence of acetone as recommended by Kober (1938). The colour was measured spectrophotometrically immediately after development and again after several hours, the difference giving them the corrected absorption due to the estrogen pink colour.

"A rather similar method has been worked out independently by Stevenson and Marrian (1947). It has been found that whereas the estrogen pink is completely discharged by heating at 100°C. for 1½ hr., the brown colour given by the total phenolic fraction of human male urine is almost unaffected by this treatment. In the determination of total estrogen in pregnancy urine, therefore, the absorption at 520 mμ is measured before and after heating, the difference giving the corrected absorption at this wave-length due to the estrogen pink.

"Bachman and Pettit (1941) attempted to avoid the necessity of a correction by eliminating from the estrogen concentrates the substances which give rise to the brown colour in the Kober reaction. By separating the estriol and the estrone-estradiol fractions and then purifying these fractions by distribution between various pairs of immiscible solvents, they obtained products which gave little brown colour, as judged by absorption at 420 mμ, in the Kober reaction.

"A somewhat similar approach to the problem has been made more recently by Stimmel (1946). The total ether-soluble phenolic material obtained from urine was separated into estriol, estrone, and estradiol fractions by adsorption on Al_2O_3 and fractional elution with different

methanol-benzene mixtures. The three fractions so obtained gave colours in the Kober reaction which showed little absorption at 420 mμ. Stimmel has suggested that the final coloured reaction mixture should be shaken with ethyl acetate before measurement of the colour. It was claimed that this procedure removed impurities without affecting the pink colour in the aqueous phase."

An example of the way in which the Kober reaction has been carried out by workers with it may be given as follows.

F. COLORIMETRIC ESTIMATION OF ESTROGENS BY THE KOBER REACTION (STEP D)

i. *Preparation of Reagent.* Kober (1931) used phenol and sulfuric acid, and in 1938 substituted β-naphthol for phenol (see also, Jayle *et al.*, 1943). However, the phenol reagent has been found quite satisfactory by most workers. This reagent was prepared by Cohen and Marrian (1934) as follows. "5.6 parts by volume of pure concentrated H_2SO_4 and 3.6 parts by volume of phenol (at 60°C.) were measured out by pipettes and mixed thoroughly in a dry well-stoppered bottle. Owing to the high viscosities of the liquids prolonged draining of the pipettes was necessary." Venning *et al.* (1937) add the following details: the phenol must be freshly redistilled and only pure H_2SO_4 should be used. The H_2SO_4 should be added slowly to the phenol, care being taken to exclude all traces of moisture and to prevent the temperature of the mixture from rising. The reagent should be kept in a glass-stoppered flask to exclude moisture and dust particles. A standard solution of pure estriol (1 ml. $\equiv$ 30 μg.) in ethanol should be used to check the efficiency of each new lot of reagent; the reagent should also be checked every week. The nature of the reagent makes accurate measurement with a pipet difficult and precludes the use of a greased stopcock. A tapless 25-ml. buret, filled from below by suction, may be used, the flow being regulated by a pinchcock on a rubber tube attached to the upper end (see also Bachman, 1939).

ii. *Colorimetric Estimation of Estrogens.* (Venning *et al.*, 1937; Stevenson and Marrian, 1947.) Measured samples of the solution of the urinary phenolic fraction, containing between 10 and 80 μg. of estrogen and equivalent to not more than 2%* of the 24-hr. urine specimen, were evaporated to dryness in a stream of air in test tubes *ca.* 2 cm. in diameter, bearing graduation marks at 8 and 15 ml. To the dry residues 3-ml. portions of the Kober reagent were added, and the tubes were heated in

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* When amounts of phenolic fraction equivalent to more than 2% of the 24-hr. urine specimen were used, precipitates were sometimes formed during the subsequent fading procedure.

the boiling-water bath for 20 min. After cooling in an ice-salt freezing mixture, 3.0 ml. of water was added to each tube and the contents thoroughly mixed. The tubes were then heated for 3 min. in the boiling-water bath, cooled to room temperature by immersion in water, and finally made up to the 15-ml. mark with 10% (v/v) H_2SO_4. The intensities of absorption at *ca.* 520 mμ were measured on 7-ml. portions of the final solutions in a Spekker photoelectric absorptiometer using an Ilford spectrum green No. 604 light filter.

The 8-ml. portions of the solutions remaining in the tubes were heated in the boiling water bath for 1.5 hr. in order to fade the pink color produced by the estrogen. After cooling and making good the water lost by evaporation, the absorption at *ca.* 520 mμ was again measured. The amount of estrogen (as estriol) originally present in each tube was obtained by referring the difference between the initial and final absorptiometer readings to an estriol calibration curve.

G. NOTES

1. The urinary extracts can be prepared as given above (steps A, B, and C) and dissolved in ethanol for evaporation in the tubes used for the color development.

2. Any suitable photoelectric absorptiometer can be used for the final estimation. A light filter passing as narrow a band of light as possible at about 520 mμ will be required.

3. As will be seen from the details, an estriol calibration curve must be prepared for the estimation, and this should be checked at some points with each new sample of reagent, and weekly.

Detailed steps A, B, and D are designed to give a complete method for Kober estimation of "total urinary estrogens." Steps A, C, and D should enable an estimation of "estriol" and "estrone + estradiol" separately to be made.

H. OTHER CHEMICAL METHODS OF ASSAY

In addition to the Kober reaction, a great many other suggestions have been made for the quantitative estimation of the estrogens by chemical or physical methods. Examples are given below.

Colorimetric methods proposed up to 1936 were examined by Pincus *et al.* (1936). While confirming (by biological assay) the value of Cohen and Marrian's (1934) technic for human urines of the sixth to ninth month of pregnancy, Pincus *et al.* (1936) regarded the Kober reaction as unreliable for urines in earlier pregnancy and for other urines. The benzoyl chloride test of Görtz (1934) had certain advantages in distinguishing between estrone and estradiol on the one hand, and estriol

on the other. The David (1934) sulfuric acid-arsenic acid test for estriol ("Marrian-crystals") was found to be inapplicable to urinary extracts. Other methods mentioned by Pincus *et al.* (1936) include the use of the ultraviolet absorption spectrum at 2800° A* (Chevallier *et al.* 1935), a suggestion by Zimmerman (1935) that his *m*-dinitrobenzene reaction could be used to estimate estrone, and the proposal by Schmulovitz and Wylie (1935) that the azo-dye formed with the natural estrogens and diazotized *p*-nitraniline should form the basis of a colorimetric assay of urinary estrogens.

Other suggestions for assay methods have been the following.

Schmulovitz and Wylie (1936) suggested coupling of estrone with *p*-diazobenzene sulfonic acid.

Shute (1938) determined the estrogenic substance in blood serum by an estimation of the "antiproteolytic power of the serum."   •

Bachman (1939a) showed that when estriol was heated at 150°C. with a reagent made up of sodium *p*-phenol sulfonate in phosphoric acid a stable violet-pink color was produced; the reaction was "specific" for estriol. The same author (1939b) proposed a modified Kober reagent for use with the three natural estrogens.

Talbot *et al.* (1940) coupled the weakly phenolic ketones ("estrone") of urine (isolated with Girard's reagent T as described above) with diazotized dianisidine. Reifenstein and Dempsey (1944) slightly modified this method, for example by using carbon tetrachloride in the continuous extractor of Hershberg and Wolfe (1940) for removal of the estrogens from the hydrolyzed urine. They also reported on the effect of impurities in various solvents (ether, toluene, CCl_4) used in the method on the final color. The method was unfavorably compared with biological assay by Bender and Wilson (1947).

Kleiner (1941) suggested that the reaction of the phenolic group of the estrogens with phthalic anhydride and stannic chloride might be made the basis of quantitative estimation.

Szego and Samuels (1943) showed that, in chosen conditions, guaiacol sulfonic acid could be used to estimate estrone + estradiol without significant interference from estriol. They then separated out the ketones with Girard's reagent T, and estimated the non-ketonic fraction, estrone being obtained by difference. The method was applied to *tissue extracts* and recovery figures, averaging 59.6% of added hormone, are reported.

Veitch and Milone (1945) showed that estrone could be determined, in pure solution, as the 2:4 dinitrophenylhydrazone.

The (characteristic) infrared spectra of the estrogens were plotted by

* See also Cheymol and Carayon-Gentil (1946).

Furchgott *et al.* (1946).   Infrared spectrophotometry has been used by
Carol *et al.* (1948) for analyses of commercial samples of estrogens.

A polarographic determination of estrone as the Girard T derivative
was worked out by Björnson and Ottensen (1946) and was applied to the
assay of estrone in the urine of pregnant mares.

Dhéré & Laszt (1947) showed that a fluorescence with neutral
dimethyl sulfate was given by estrone, estradiol, equilin, equilenin, and
other steroids.

Finkelstein *et al.* (1947) used a method based on the green fluorescence
produced when the steroid estrogenic hormones are heated with phos-
phoric acid.   Finkelstein (1948) has applied this method to estimations
of the estrogens separated from urine and claims considerable sensitivity
and accuracy for it.

Cohen and Bates (1947) omitted the phenol from the Kober reagent,
simply heating the estrogens with sulfuric acid and measuring the color
with a filter passing light at 510 mμ.   The method has also not yet been
applied to urinary extracts.

Jailer (1948) also observed that the fluorescence produced when sul-
furic acid was heated with solutions of estrone and estradiol could be
used for quantitative estimation.   Quite concordant results were obtained
between biological assay and the new method as applied to urinary
extracts and recoveries of about 45–55% of estrone and estradiol added
to urine were reported.   Clinical applications of the method were investi-
gated, and reports on its value from other laboratories will be awaited
with interest.

Salter *et al.* (1948) have used a "tentative" Kober method for which
they claim results of value in the examination of what are called the
urinary "estroids" of non-pregnant women and in selected clinical cases.
It is emphasized that the results have only relative significance, and
indeed the very uncertain recovery figures quoted with the method make
it clear that a chemically reliable procedure has not yet been evolved.
Such reliability is not, in fact, claimed, but the work of Salter *et al.* is
clearly a valuable addition to our experience of the Kober estimation.

In a recent article Friedgood and Garst (1948) have given an account
of a careful examination of the method of identification and quantitative
estimation of the three common natural estrogens by ultraviolet absorp-
tion spectrophotometry.   In this article, Friedgood and Garst criticize
the use of sodium carbonate for the separation of estriol from estrone and
estradiol, as used by Bachman and Pettit (1941), Mather (1942), and
Pincus (1945), stating that "approximately one-third of the estradiol is
carried over into the alkaline phase with the estriol."   If this is true,
it will not greatly affect the value of the Bachman and Pettit method for

pregnancy urine, as estradiol is in any case only a minor constituent in this source. Friedgood and Garst prefer to use sodium phosphate solution at about pH 8.7 as a medium for the separation of estriol, although their choice is to some extent based on considerations of spectrophotometry. The work of Friedgood and Garst is valuable especially for the thoroughness with which they have investigated the possible difficulties in its application to urine, etc., and its development for the assay of biological material will be awaited with interest. It may be pointed out, however, that, as Friedgood and Garst themselves are clearly aware, the absorption spectrum of the natural estrogens is largely due to the phenolic grouping in their molecules and is therefore not of itself much more specific than most of the chemical color tests; indeed it may actually be less specific than the Kober reaction. For other details, see Friedgood et al. (1948).

2. Some Results

Summaries of the more important findings, both by biological and chemical assay, of estrogen concentration in urine and in tissues have been given elsewhere, e.g., by Robson (1947), and it will therefore not be necessary to repeat this information here. However, a word must be said as to the particular significance of chemical assay. There is very serious doubt as to the reliability of the chemical assay methods, except possibly, as applied to human urine of late pregnancy, in which the estrogen excreted may be as much as 10–20 mg./24-hr. specimen, and even in this case, overestimation by chemical assay is common. Thus, for example, Smith et al. (1939) state "until some more specific color reagent for the estrogens is found, we are forced, therefore, to depend entirely upon bio-assay in measuring the estrogenic content of urines from both pregnant and non-pregnant women."

Bender and Wilson (1947) give their opinion that "no chemical method is yet available for the estimation of estrogens in normal urine and the biological assay remains the only reliable method."

Writing about the Kober methods, Marrian (1948a) sums up the position as follows. "It is doubtful whether any of the published methods of correcting for or eliminating the brown colour produced by non-estrogenic substances are reliable when less than about 2 mg. estrogen/24 hr. are present in the urine. Since biological assays indicate that the maximum estrogen excretion during the menstrual cycle is of the order of 0.1 mg./24 hr. the value of these colorimetric methods for the determination of estrogen in the urine of non-pregnant women is somewhat questionable. However, Jayle et al. (1946) have recently applied their

method to the daily determination of estrogen excretion throughout the menstrual cycle, and, although their figures may not have much quantitative significance, it is of very great interest that their excretion curves closely resemble in shape those obtained by other workers using biological methods of assay. These results suggest that further work directed towards improvement of methods might in the future lead to a reliable colorimetric procedure for the determination of estrogen in the urine of non-pregnant women."

Values for human pregnancy urine are perhaps more reliable and are quoted, e.g., by Cohen *et al.* (1935), Bachman (1941), and by Stimmel (1946), as well as many other workers, e.g., Robson (1947), Venning (1948).

Attention must be drawn to values quoted, e.g., by Cohen *et al.* (1935) and by Hain (1938, 1939, 1942), for what are referred to as "free" and "combined forms" of estrogens. By "free" estrogen is meant that which can be extracted from urine with ether, benzene, etc. before hydrolysis. Although Cohen *et al.* (1935) state that their method of collection of urines was such that "any bacterial hydrolysis . . . was prevented," their description of the conditions of collection and preservation of urine are by no means convincing in this respect, whilst Hain (1938) does not mention any precautions against bacterial action. It has recently been realized that the preservation of urine, and especially urine of pregnancy, from bacterial growth is a very difficult matter indeed, and decomposition, e.g., of glucuronide, may occur in a surprisingly short time (see Bucher and Geschickter, 1940). Barber *et al.* (1948) have found that certain strains of *Staph. albus* will readily hydrolyze urinary glucuronide, and the present author has encountered cases in which 24 hourly urine collections, received in the laboratory 1 hr. after the end of the collection, and then immediately extracted with butanol, yet contained no measurable butanol-extractable glucuronide whatever. Since estriol is excreted as the glucuronide and since estriol is apparently the chief estrogen in urine of late human pregnancy, e.g., Cohen *et al.* 1935; Hain, 1939; Stimmel, 1946, it seems clear that determinations of "free" estrogen in such urine ought to be carried out with very careful precautions indeed against bacterial hydrolysis. These remarks do not of course imply that "free estrogen" is not excreted at all in urine of pregnancy. They are intended to emphasize that, when published work is considered, great reliance cannot apparently be placed on the quantitative values quoted for "free" estrogen excreted in urine.

Marrian (1948b) has expressed the opinion that "free" estrogen might possibly be formed as the result of an unusually high concentration

of glucuronidase in urine of patients in labor; he does not think that his earlier results can be entirely explained by bacterial decomposition (see Clayton and Marrian, 1950).

Mayer *et al.* (1940) have applied a Kober method to the assay of estrogen in the follicular fluid as well as the urine of non-pregnant and pregnant mares.

3. Estimation of Synthetic Estrogens (Summary)

Diethylstilbestrol (4:4'-dihydroxy-α,β-diethylstilbene). Elvidge (1939) proposed estimation by absorption spectrophotometry, Sondern and Burson (1942) recommended a volumetric analysis, whilst colorimetric assays have been worked out by Dingemanse (1940), Dechne (1941), Tubis and Bloom (1942), Huf and Widman (1942), Cocking (1943), Dracass and Foster (1943), and Malpress (1945). See also Gottlieb (1947).

Hexestrol (4:4'-dihydroxy-γ,δ-diphenyl-n-hexane). Absorption spectrophotometry was proposed by Elvidge (1939) and Goetze and Seif (1945), whilst Malpress (1945) has put forward a colorimetric method.

Dienestrol (4:4'-dihydroxy-γ,δ-diphenyl-$\Delta^{\beta,\delta}$-hexadiene). Malpress (1945) has recommended a colorimetric procedure.

The antimony pentachloride reaction of Dingemanse (1940) and the reaction based on nitration of the estrogens (Malpress, 1945) were investigated by Warren *et al.* (1948). These authors tested the reaction of a number of synthetic estrogens (substituted stilbenes) with antimony pentachloride. Warren *et al.* could not demonstrate free or conjugated stilbestrol in the urine of subjects undergoing treatment with large therapeutic doses of this compound.

III. PREGNANEDIOL

This compound was isolated by G. F. Marrian in 1929 from human pregnancy urine. Its structure (I) was elucidated by Butenandt (1930, 1931). Odell and Marrian (1936) showed that pregnanediol existed in human pregnancy urine as an acid-hydrolyzable compound which could be extracted from the urine with *n*-butanol. Venning and Browne (1936) showed that this complex was pregnanediol glucuronidate, the structure of which (II) was proved by Heard *et al.* (1944). The glucuronidate was finally synthesized from pregnanediol by Huebner *et al.* (1944).

The structure of pregnanediol is clearly closely related to that of progesterone (III), and pregnanediol is in fact easily converted into progesterone in the laboratory. The metabolic relationship between

pregnanediol and progesterone is likewise quite clear, and the subject has recently been reviewed by Marrian (1947).

It has also been shown that administered deoxycorticosterone can be converted into pregnanediol in man (see below), the chimpanzee (Horwitt *et al.*, 1944), and the rabbit (Hoffman *et al.*, 1943).
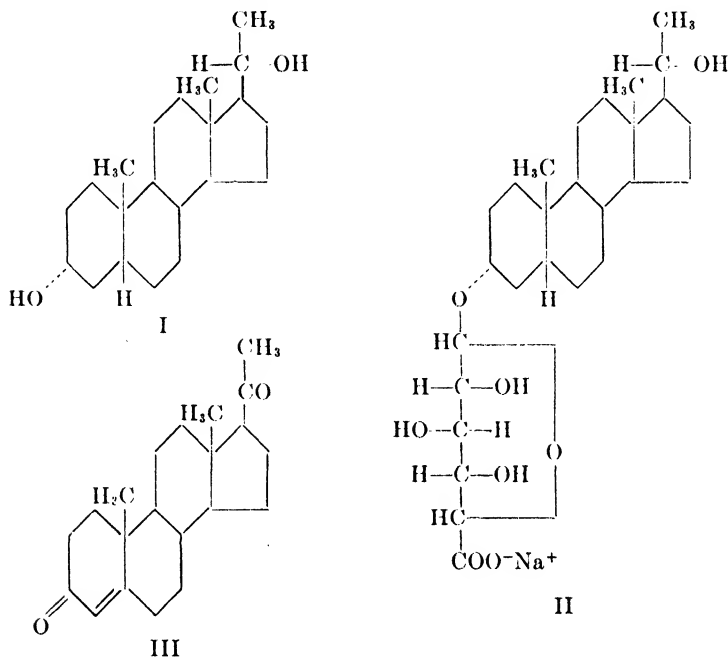
With the recognition of these relationships, methods of determination of pregnanediol in human urine have been much studied. Such methods can be divided into two types: (1) those in which the aim is to estimate the pregnanediol glucuronide; (2) those in which "free" pregnanediol is estimated after hydrolysis of the complex.

1. Estimation of "Combined" Pregnanediol

The best-tried method of estimation of pregnanediol as sodium pregnanediol glucuronidate (NaPG) is that of Venning (1937, 1938), and is given in full below.

A. GRAVIMETRIC ESTIMATION OF SODIUM PREGNANEDIOL GLUCURONIDATE IN HUMAN URINE (VENNING, 1937, 1938)

A 24-hr. specimen of urine (preserved, see Note i) is collected, measured, and an aliquot amount is taken which may be expected to contain 10–15 mg. of sodium pregnanediol glucuronidate (NaPG), i.e., 6–9 mg. of

pregnanediol.   In urine of the menstrual cycle it is necessary to extract the full 24-hr. or even 48-hr. specimen.

The urine is extracted in a separating funnel 4 times with a total of about one-third of its volume of n-butanol.   (If 1 liter of urine has been taken, the volumes of butanol are 200, 75, 50, and 50 ml.)   The mixture should not be shaken vigorously but gently mixed with a rotatory motion, each extraction requiring approximately 1 min.   Some urines nevertheless do form an emulsion and, in order to separate the butanol, the mixture may be centrifuged.   Formation of an emulsion tends to cause a loss of NaPG unless the urine separating out from the centrifuging of the emulsion is again extracted with butanol.

The butanol should be clear before it is evaporated.   If there is a residue at the bottom, the clear butanol is poured off and the residue extracted with fresh butanol, which is added to the first fraction.   The whole of the butanol is then taken to dryness under reduced pressure. When a suitable vacuum pump is used and the evaporation flask is kept in a boiling-water bath, the butanol distils over rapidly (but see Note ii) and the temperature of the boiling butanol should remain between 40–49°C.   The residue in the flask is then dissolved in 60 ml. of 0.1N NaOH.   This alkaline solution is extracted 4 times with butanol (20, 20, 10, and 10 ml.).   The combined butanol fractions are allowed to stand until clear, as some residue usually settles out.   The clear butanol is poured back into a separating funnel, and the residue is washed twice with small amounts of butanol, which are added to the funnel.   The combined butanol is finally washed once with 3 ml. of 0.1N NaOH and then twice with 5 ml. of water.   After each washing, the mixture is allowed to stand or is centrifuged for complete separation of the layers. The butanol is again evaporated to dryness under reduced pressure. Exactly 5 ml. of water is added to the residue with about 10 ml. of acetone.   The residue is dissolved in this mixture and transferred to a 125-ml. conical flask, the original flask being washed out several times with acetone.   The final volume of the mixture is made up to 100 ml. with acetone, and it is allowed to stand overnight at 5–10°C.   A precipitate settles out.   Most of the supernatant fluid can be drawn off by suction, and the remainder, with most of the precipitate, is transferred to a suitable tube and centrifuged.   The supernatant acetone is then poured off without disturbing the precipitate, which is left in the tube. A second precipitation with acetone is made, and for this purpose the precipitate is redissolved in water.   The amount of water depends upon the weight of the precipitate, which has to be approximated.   If it is 10 mg. or more, 5 ml. of water; if 5–10 mg., 3 ml. of water; and if less than 5 mg., 2 ml. of water is used.   The water is measured into the original

conical flask (which still contains some precipitate not originally transferred to the centrifuge tube), and an equal volume of acetone is added. The mixture is warmed to dissolve the precipitate still adhering to the flask, the solution is transferred to the original centrifuge tube, and the precipitate in this is dissolved by heating. The solution is cooled and filtered (if necessary) back into the conical flask, with suction. On cooling, a considerable amount of impurities may come out of solution while the small amount of NaPG remains dissolved. Filtering the mixture at this stage is of particular value in the case of urines from the menstrual cycle; however, when the first precipitate is relatively pure, as in the case of pregnancy urine, it may be omitted. Acetone is used to wash out the filter (if used) and the centrifuge tube, and the final volume in the conical flask is made up to 100 ml. with acetone, as before.

After standing overnight in the refrigerator, the supernatant fluid is again drawn off, and the remainder is centrifuged. If the precipitate does not settle down completely but remains in suspension, the remaining fluid should be filtered. The whole of the precipitate (in flask, centrifuge tube, filter) is dissolved in hot ethanol. Occasionally a small amount of water has to be added to ensure complete solution of NaPG. The hot alcohol is filtered (if necessary) into a weighed beaker, the filter, etc. being washed with further hot ethanol. The contents of the beaker are evaporated to dryness on a water bath, and the weight of the residue is obtained. A melting point is taken (see Note iii).

B. NOTES ON (A)

i. *Preservation of Urine.* This is of the greatest importance if the Venning method is to give reliable results. Venning (1938) herself states that tricresol, chloroform, or toluene are unsatisfactory at temperatures of 18–34°C. Sodium cyanide (0.5%) would prevent hydrolysis for 4 days at 20°C., but not at 26–34°C. Bucher and Geschickter (1940) reported inaccurate determinations of NaPG with incompletely preserved urine and compared "conjugated" and "free" pregnanediol. They, too, reported the failure of tricresol (6 drops per 24-hr. specimen) to preserve the urine in summer weather, and recognized that bacterial action was probably responsible for losses of NaPG, especially as these losses did not occur with sterile catheter specimens in which, they state, "free" pregnanediol could not be found.

Allen and Viergiver (1941) used butanol for preserving pregnancy urine. They do not cite any experiments designed to examine its usefulness for this purpose. Jayle *et al.* (1943) used 0.1 g. mercuric cyanide per 24-hr. specimen. They do not discuss the efficacy of this preservative.

Bisset *et al.* (1948) found that if urine was voided directly into a bottle containing 3 g. of a 2:1 (w/w) mixture of finely powdered thymol and salicylic acid and was then shaken, apparently complete temporary preservation could be obtained. Even urine from a subject in the ninth month of pregnancy did not appear to lose "pregnanediol-like glucuronide" (see below) until after 48 hr. at 37°C., and in none of 10 samples did visible growth of microorganisms appear in 48 hr. at 37°C. There seems to be no reason why this method should not be applied in the Venning analytical procedure.

ii. *Purification of n-butanol.* Bisset *et al.* (1948) have found that some specimens of *n*-butanol destroyed NaPG with which they were evaporated at reduced pressure. They distilled commercial *n*-butanol before use over potassium hydroxide (5 g./l.) and collected the fraction which came over in a fractionating column at 112–118°C. This was then redistilled at *ca.* 70°C./20 mm. Hg with an air-leak and in the presence of 1 g./l. of 4-amino phenol and 1 ml./l. of $5N$ NaOH. Finally the butanol was redistilled at 60–70°C./20 mm. Hg. The same authors did not allow the water bath temperature to rise above 55°C. when the butanol extracts of urine were evaporated at *ca.* 20 mm. Hg pressure. It is of course well known that *n*-butanol may undergo oxidation on standing under laboratory conditions, but it is not clear whether it is this process or some original impurities which provide the substances destructive of NaPG.

iii. *Nature of Venning's Precipitate.* Since Venning published her method, it has been shown that "NaPG" obtained by it is in fact a mixture of glucuronidates.

Sutherland and Marrian (1947) separated pure NaPG ($+3H_2O$, m.p. 283.5–284.5°C. corr., with decomp. and evolution of gas) from the Venning material and showed that the precipitate probably contained about 80% of non-ketonic substances. The glucuronidate of pregnane-3α-ol-20-one was the chief ketone present (Marrian and Gough, 1946), and an impure preparation of this had m.p. 257–260°C. corr. (with decomp. and evolution of gas).

Glucuronidate obtained by essentially Venning's procedure from a case of adrenal cortical hyperplasia by Mason and Kepler (1945) had m.p. 250–253°C. and yielded, after enzymic hydrolysis, pregnane-3α,17,20-triol, as well as pregnanediol and a ketonic mixture. Mason and Strickler (1947) obtained a sodium glucuronidate fraction, m.p. 266–268°C. from the urine of a female pseudohermaphrodite. This substance after hydrolysis yielded pregnane-3α,17-diol-20-one.

It would seem therefore, that the classification of glucuronidate isolated by the Venning method as "NaPG" is quite unjustified, as is

the statement that the weight of Venning precipitates (of whatever melting point) can be translated directly into "pregnanediol."

iv. *Possible Shortening of Procedure.* Jayle *et al.* (1943) adjusted the urine to pH 10 with sodium hydroxide before extraction, used a single extraction with butanol, and applied a factor to allow for losses of NaPG thus encountered.

v. *Further Remarks.* It is essential to bear the above qualifications about the nature of the Venning precipitate in mind when one is considering the results of an analysis. However there is no reason to suppose that the results obtained so far by the method are meaningless because of uncertainty about the nature of the glucuronidate estimated. On the contrary, it may well be that it is the arbitrary relation of the excretion of pregnanediol alone to luteal or adrenal cortical function which may cause the more serious error in interpretation. The matter is further discussed below.

As pointed out by Venning herself, the above method of analysis is particularly well adapted to urines containing not much less than about 10 mg. of NaPG per 24-hr. specimen. At lower levels of excretion, the final crystallization from acetone becomes difficult because of the accumulation of impurities arising from the large volume of urine which has to be extracted in order to obtain enough NaPG for weighing.

Venning (1938) states that "it was impossible to detect a total amount of 1 mg. of NaPG at any time" when this was added to 1 liter of urine. With 2 mg. added, recovery was 60%, with 3 mg., 67%, and with 5 mg., 74%. Even with larger amounts of NaPG, recovery was not quantitative (Venning, 1937), although sufficient experiments are not reported to enable one to make a statistical assessment of the accuracy of the method. In any case, at that time, pure NaPG was unknown; furthermore, accurate recovery experiments with it added to, say, male urine would have little meaning, as applied to the assessment of the method in actual use, where, as discussed above, substances other than NaPG are encountered. The method has, in practice, given very valuable results in pregnancy and has proved capable of at least detecting the NaPG excreted in the luteal phase of the human menstrual cycle. It has also been used successfully to detect pregnanediol formed from progesterone administered to women and also from administered deoxycorticosterone. The Venning procedure is of particular value as a general method of obtaining solid glucuronidates from urine, e.g., Mason and Strickler, 1941; Mason and Kepler, 1945, and should never be neglected when there is a question of the possible excretion of an unusual steroid as glucuronidate. Its general application to animal urines will be awaited with much interest.

Nevertheless, when all this has been said, it remains true that the method is not a particularly sensitive one and is likely to fail to estimate with reasonable accuracy amounts of NaPG of the order of those sometimes excreted in 24 hr. in the luteal phase of the human menstrual cycle. It is not surprising therefore that other attempts should have been made to determine pregnanediol, as glucuronidate, by quite different methods, especially since the estimation of luteal pregnanediol in the normal cycle is of importance as a method of deciding whether ovulation has occurred.

Westphal (1944) claimed to have improved the sensitivity and specificity of the Venning method by a final identification of the pregnanediol glucuronide as the barium salt (m.p. 272°C. with decomposition), which separated as characteristic crystals when barium acetate in 50% ethanol was added to NaPG, in the same solvent. With this method, Westphal was able to detect 0.5–1.0 mg. of NaPG per liter of urine. He was not able to isolate the barium salt from female "follicular" urine, but in male urine he found amounts of pregnanediol corresponding to about 0.7 mg. of barium pregnanediol glucuronidate per liter. The method would seem to be of real value as a means of identifying NaPG when a mixture containing it has been isolated, but does not appear to offer any outstanding quantitative advantages (see, however, below).

Allen and Viergiver (1941) and Woolf et al. (1942) extracted the glucuronidate from urine with n-butanol as Venning had done. They re-extracted an alkaline solution with fresh butanol, evaporated an aliquot of the butanol and dissolved the residue in 0.1N sodium hydroxide, evaporated a butanol extract of this to dryness, and dissolved the residue in water. From this solution glucuronidate was precipitated with lead nitrate. The reducing power of the precipitate, digested with sodium carbonate solution, was estimated, acid hydrolysis was carried out, and reducing power was re-estimated. The difference was considered to be largely due to the reducing groups of glucuronic acid molecules liberated by the hydrolysis. Good recoveries of NaPG from simple solution were obtained, but the authors found that female "follicular" urine contained substances which interfered with the estimation, giving fictitiously high results for NaPG. This finding is not at variance with what is now known about "steroid glucuronides" (see below) in human urine, but Allen and Viergiver considered their method not valid for estimation of NaPG in urines other than those of pregnancy. To pregnancy urines they applied their method, obtaining results very similar to those of users of the Venning analysis and finding good recoveries of NaPG added to such urines.

Using the method of Allen and Viergiver and preserving the urine by collection under n-butanol, Woolf et al. (1942) examined the dis-

tribution of NaPG between *n*-butanol and (1) "neutral" (native) urine and (2) urine made strongly alkaline with sodium hydroxide. They found that NaPG (Venning) was 13 times as soluble in *n*-butanol as in the neutral urine and 12 times as soluble in butanol as in the alkaline urine. It should be possible, therefore, to calculate the total amount present in a given sample of urine from the amount actually obtained by a single estimation with approximately one-third volume of butanol. The work of Woolf *et al.* is interesting as indicating the great efficiency of the butanol extraction.

Hechter (1942) compared the weights of precipitates obtained by the Venning method with their reducing power, after acid hydrolysis, as determined by the Folin-Wu colorimetric blood sugar technic. Hechter found general agreement between the gravimetric and reducing power analyses, except that in two patients, when the Venning method gave positive values up to 6.7 mg./24 hr. in absence of luteal activity as shown by a vaginal smear, the determination of potential reducing power indicated that the precipitates were 85–100% impure (not glucuronidate).

C. THE NAPHTHORESORCINOL REACTION

Crismer (1939), Jayle *et al.* (1943), and Jayle and Libert (1946) have applied the Tollens naphthoresorcinol reaction to the estimation of NaPG. This reaction depends on the formation of a pigment, soluble in organic solvents but not in water, when glucuronic acid is heated in acid solution with partially oxidized naphthoresorcinol (1,3-dihydroxynaphthalene). Glucuronides can be hydrolyzed by the heating with acid, and the glucuronic acid set free reacts at once with the naphthoresorcinol reagent. This reaction is given by uronic acids in general, and pigments are also formed by certain sugars and other substances. Nevertheless, sensitivity is great, and the color of the pigment is sufficiently characteristic to enable one to detect easily when interference by substances other than uronic acids occurs.

Jayle and his colleagues isolated the glucuronidate from urine by a modification of the Venning extraction method and evaporated the (washed) butanol extracts to dryness. To the residue from one such process they applied directly the naphthoresorcinol reaction, another residue was precipitated with acetone as in the Venning method, and a third residue was dissolved in methanol (1 ml.) and precipitated with ether (27 ml.). To each precipitate the naphthoresorcinol reaction was applied. Figures were thus obtained for "direct estimation," "acetone crystallization," and "ethereal precipitation." Examples of such figures are given in Figs. 1 and 2.

It will be observed that according to Jayle *et al.* the results of the

"direct" estimation are parallel to but higher than the figures obtained with Venning's NaPG. If these results are accepted as correct, they suggest that the elaborate precipitation technic of Venning may be unnecessary; all that the analyst need do is to apply the naphthoresorcinol reaction to the residue from the evaporation of suitably washed butanol
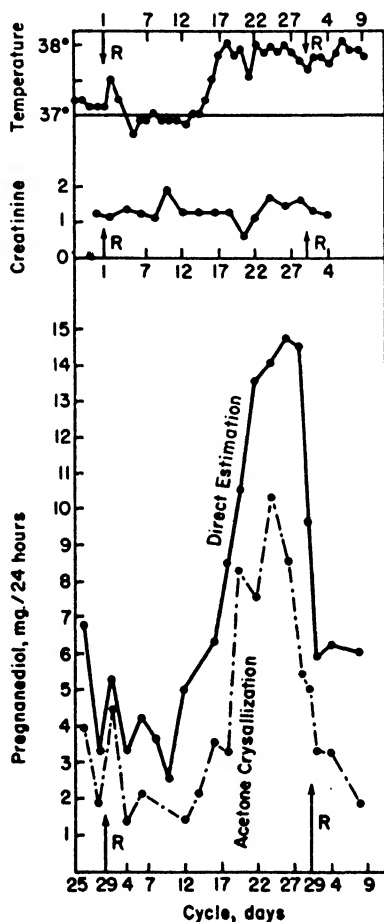
FIG. 1.   Pregnanediol in the menstrual cycle (Jayle and Libert, 1946).

extracts. The figures obtained are likely to have at least as much *clinical* value as those obtained by the complete Venning analysis.

One difficulty not mentioned by the French authors is that of the actual application of the naphthoresorcinol reaction to these butanol residues. The present author's experience has been that substances which are apparently not glucuronides but which interfere seriously with

the naphthoresorcinol reaction are present in such residues.   The position is further complicated by the fact that Jayle *et al.* give no details of the color reaction which they used.

Jayle *et al.* also showed that the 24-hr. collection of urines could be dispensed with, in individuals whose creatinine excretion could be assumed constant, by expressing NaPG as milligrams per gram of creatinine (Fig. 2).

The French workers applied the term "steroid glucuronides" to the fractions which they estimated as described above and thus clearly recognized the indefinite nature of the complex containing pregnanediol glucuronidate.
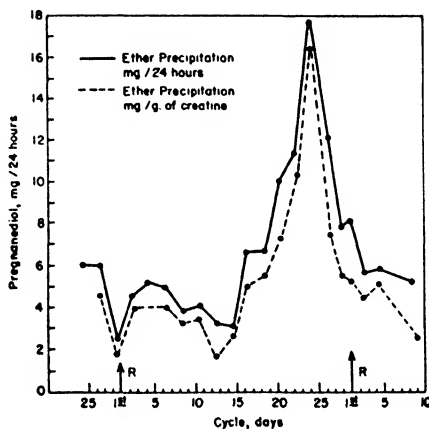
Fig. 2.   Steroid glucuronides in the menstrual cycle (Jayle and Libert, 1946).

Similar recognition is implied in the term "pregnanediol-like glucuronide (PLG)" applied to a fraction, isolated from urine by Bisset *et al.* (1948) by an entirely different process.   These authors "entrained" glucuronidate (presumably as barium salt) on a precipitate of barium phosphate, dissolved the washed precipitate in dilute hydrochloric acid, and applied a modified naphthoresorcinol reaction to portions of the solution.   Glucuronidate was extracted from urine with *n*-butanol as in the Venning method, and the residue from the evaporation of the washed butanol was dissolved in water or alkali for entrainment.

The results obtained by this method in urine of the menstrual cycle and in pregnancy were similar to those reported by Jayle *et al.*, and higher than those found by the Venning analysis.   In male urine, PLG excretion was about 4–7 mg./24 hr., and it is clear that this can include only very little NaPG.   Assessment of the possible value of the method and of the conception of PLG must await further experimental work.

Since the naphthoresorcinol reaction is one very useful in the study of urinary glucuronide, special attention was given to its application to NaPG by Bisset *et al.* Maughan *et al.* (1938) had improved the sensitivity of the original Tollens test by using an aqueous instead of an alcoholic solution of naphthoresorcinol. They extracted the colored substance formed with ethanolic ether and measured the color with a photoelectric colorimeter. The reaction was actually applied to recovery of NaPG from urine by these authors.

Hanson *et al.* (1944) preferred amyl alcohol as a solvent for the colored product of the reaction. They also investigated its efficiency for a number of differential glucuronides. Bisset *et al.* (1948) again used ethanolic ether and further improved the reaction by introducing a simple method of partially oxidizing the naphthoresorcinol solution with a little potassium ferricyanide, thus eliminating the previously used tedious and uncertain "aging" process.

D. THE NAPHTHORESORCINOL REACTION AS APPLIED TO URINARY NaPG (BISSET *et al.* 1948)

Naphthoresorcinol solution. A small excess over the amount of naphthoresorcinol needed for the day's experiments is weighed out and dissolved at about 50°C. in water to make a $0.025M$ solution (0.4 g./100 ml.), which is then filtered. Quantities are measured into wide test tubes (15 × 2 cm.) as follows.

Test and Standard (Duplicated)	*Blank*
(1) Glucuronide solution (containing not more than 0.2 mg. NaPG) + water to make 1.4 ml.	1.4 ml. water
(2) 0.1 ml. $0.05M$ potassium ferricyanide	0.1 ml. $0.05M$ potassium ferricyanide
(3) 3.5 ml. $5N$ HCl	3.5 ml. $5N$ HCl
(4) 2.0 ml. naphthoresorcinol solution, as above	2.0 ml. naphthoresorcinol solution

The "standard" contains a known amount of NaPG (0.1 mg. or 0.15 mg. are suitable quantities).

The contents of each tube are thoroughly mixed, and small funnels are placed in the mouths of the tubes, which are then suspended by rubber bands clear of the bottom of a water bath. The water is heated *to 100°C.* for 2.5 hr., the contents of each tube being again mixed shortly after heating has begun. The tubes are now cooled in ice, and the contents of each in turn poured and washed into a small separating funnel with 14 ml. of ethanolic ether (20% w/v ethanol/peroxide-free ether). The stoppered funnel is *vigorously* shaken and, after separation, the

aqueous portion of the contents is run off. The colored extracts are poured as quickly as possible from the mouth of the funnel into colorimeter tubes which are immediately stoppered. The colors, which are stable for some time in absence of strong daylight, are measured in a suitable photoelectric colorimeter, with a green or yellow filter.

The reading for the blank is subtracted from that for the test and standard, and the amount of NaPG in the test calculated in the usual way.

With this method, Beer's law has been found to be quite closely obeyed up to 0.2 mg. of (pure, anhydrous) NaPG. The results were closely reproducible (8 readings with 0.1 mg. Venning's NaPG gave E, 0.2795 (average), σ of an individual reading, ± 0.0076).

For a standard, Venning's NaPG can be used. Bisset *et al.* found that 1 mg. of a sample of this was equivalent to 0.777 mg. of anhydrous NaPG, by reference to a pure specimen, prepared by Marrian and Gough (1946). This figure cannot of course be taken as more than indicative of the sort of relationship, which exists between Venning's material and pure NaPG. Thomson's gray solution (1946) was also tested as an artificial standard. When this was diluted to twice its volume with water the ratio gray solution (colorimeter readings)/0.078 mg. pure anhydrous NaPG (colorimeter readings *less* blank) was 1.24 (average of 17 separate experiments), σ of an individual reading ± 0.06. An Ilford Spectrum yellow light filter was used.

The ethanolic-ethereal colored extracts were found to have almost consistent light absorption between 560 and 600 mμ, but the color altered on exposure to strong daylight.

The carbazole-sulfuric acid reaction of Dische (1947) for estimation of glucuronic acid has not yet been applied to the analysis of NaPG. If it proves possible to overcome the difficulty of using strong sulfuric acid with mixtures containing variable amounts of substances which give colors on treatment with this reagent, the carbazole reaction should be of great value. The procedure is very much simpler than that of the naphthoresorcinol process.

2. Estimation of "Free" Pregnanediol

Bucher and Geschickter (1940) showed that it was possible to estimate the free pregnanediol, if any, in the acetone used in the Venning method for precipitating NaPG. This "free" pregnanediol was added to the "combined" material calculated to be present in the precipitate, and the "total" pregnanediol thus obtained.

Astwood and Jones (1941) abandoned entirely the idea of attempting to determine combined pregnanediol. They hydrolyzed the urine by

boiling with hydrochloric acid in the presence of toluene, into which the pregnanediol passed as it was liberated from the glucuronide by acid hydrolysis. The toluene layer was then separated, and the urine was re-extracted twice with fresh toluene. The total toluene extract was washed with water and evaporated to half its volume, after the addition of methanolic sodium hydroxide. The gelatinous precipitate thus produced was filtered off, and the toluene was taken to dryness. The residue was dissolved in hot ethanol, and pregnanediol, if present, was precipitated by the careful addition of $0.1N$ sodium hydroxide. When the mixture had been allowed to cool to room temperature and had been standing in a refrigerator overnight, the precipitate was collected, reprecipitated (sometimes twice) from ethanol with water instead of $0.1N$ sodium hydroxide, transferred to a tared vial, weighed, and a melting point was taken. Astwood and Jones regarded the determination as satisfactory if the product melted at or above 220°C. or if the melting point was not depressed by admixture with pure pregnanediol. Good recoveries of NaPG added to urine were not always obtained, and Astwood and Jones, who were very critical about their own work, could not account for a "constant" loss of about 20%, for which they applied a correction in their final calculation. These authors pointed out the great advantages which were obtained by the stability of free pregnanediol and by eliminating the necessity of preserving the urine. Astwood and Jones also detected some interference by other substances, as indicated by the melting points of their final precipitates.

Talbot *et al.* (1941) combined the determination of pregnanediol and that of 17-ketosteroids on the same urinary specimen. The urine was hydrolyzed with hydrochloric acid and at the same time continuously extracted with carbon tetrachloride. After evaporation of most of this solvent the residue was dissolved in ethyl acetate, and this was washed with small amounts of N sodium hydroxide and then with water. Complete evaporation of the ethyl acetate now left a residue which was separated into ketonic and non-ketonic (neutral) steroids by treatment with Girard's reagent T. The non-ketonic steroids were treated in toluene solution with alkali and then precipitated from ethanol with $0.1N$ sodium hydroxide exactly as described by Astwood and Jones. Talbot *et al.*, instead of weighing the final precipitate, washed it with light petroleum and transferred it to a small flask with hot ethanol. The solvent was evaporated to dryness, and the residue was dissolved in fresh ethanol. An aliquot of this was evaporated to dryness in a colorimeter tube, and the residue was treated with concentrated sulfuric acid. The yellow color produced with pregnanediol was compared with that obtained from a known amount. Talbot *et al.* obtained recoveries very

similar to those reported by Astwood and Jones, and again applied a correction for the large "constant" loss of pregnanediol thought to be encountered when NaPG was hydrolyzed.

Sommerville *et al.* (1948) made an intensive study of the Astwood-Talbot procedure. They point out that some at least of the losses mentioned above can be explained by the fact that NaPG contains only about 80% of pure sodium pregnanediol glucuronidate (Marrian and Gough, 1946); other possible sources of error were examined. Sommerville *et al.* found that one important factor was the rate of cooling during the Astwood and Jones precipitation process, which if not carefully controlled might involve serious losses, especially if small amounts of pregnanediol were being estimated. Other improvements in technic were introduced, chiefly to facilitate routine working and to remove interfering substances. The method is now given in detail because, although it does not yet seem to have been applied extensively for clinical and physiological studies, it represents the experience of at least three independent groups of careful workers and is, in its final form, probably the most precise method of pregnanediol determination available at the present time.

A. QUANTITATIVE DETERMINATION OF "FREE" PREGNANEDIOL IN URINE (SOMMERVILLE, GOUGH, AND MARRIAN, 1948)

i. *Apparatus and Materials.* Glassware, with interchangeable standard glass joints, was used throughout in order to avoid contamination of the urinary extracts with colored or chromogenic material that might be dissolved out of rubber or cork stoppers.

The toluene used for the extractions was sulfur-free and was distilled before use. Ethanol was purified by refluxing over sodium hydroxide and distilling twice.

Pregnane-3α,20α-diol was prepared from human pregnancy urine and purified via its diacetate. The sample used in the recovery experiments melted at 236–237°C. (corr.).

Sodium pregnane-3α,20α-diol glucuronidate was prepared from human pregnancy urine by the method of Venning and Browne (1936) and freed from ketonic glucuronides by the method of Sutherland and Marrian (1947). The preparation used in the recovery experiments melted at 282–283°C. (corr.) with decomposition and evolution of gas. Samples of the glucuronidate were weighed out after exposure to moist air. As shown by Sutherland and Marrian (1947) material so treated is the *trihydrate* having the composition $C_{27}H_{43}O_8Na \cdot 3H_2O$.

ii. *Method.* A 24-hr. specimen of urine collected with 5 ml. of toluene as preservative is made up to 2.5 l., and duplicate 500-ml. samples are

removed. Each sample is treated as follows. It is placed in a 1-l. flask, and after the addition of 100 ml. of toluene brought to boiling point under a reflux condenser. To the boiling mixture is added down the condenser 50 ml. of concentrated hydrochloric acid (A.R.), and the boiling is continued for exactly 10 min. The flask is then rapidly cooled in cold water, and the contents are transferred to a separating funnel of 750 ml. capacity. After shaking and allowing the urine layer to separate, the latter is run off into the original flask and the layer of toluene and emulsion filtered with gentle suction through a Whatman No. 1 paper on a Buchner filter funnel. The urine layer is then returned to the separating funnel and extracted twice more with 100-ml. portions of toluene, each toluene and emulsion layer being filtered in succession through the same filter funnel. The combined filtrates are then transferred to a clean separating funnel, and after running off the small urine layer that separates, the toluene extract is washed twice with 100-ml. portions of N sodium hydroxide and twice with 100-ml. portions of water. The washed toluene extract is run into a 500-ml. round-bottomed flask and is evaporated nearly to dryness on an electric hot plate and then taken completely to dryness under reduced pressure on a boiling-water bath.

The dry residue is transferred quantitatively with warm ethanol to a 20-ml. conical centrifuge tube, and the ethanolic solution is evaporated to dryness in a water bath under a stream of air. To the residue in the tube is added exactly 4.0 ml. of ethanol, and the tube is placed in a beaker of water maintained at 75°C. After stirring with a glass rod for 1 min. to obtain complete solution, 16.0 ml. of 0.1N sodium hydroxide is added drop-wise from a buret during 3 min. with stirring, the last 1 ml. being used to wash down the stirring rod into the tube. After a further 1 min. at 75°C., the beaker of water containing the tube is transferred to an incubator at 37°C. and left overnight. Approximately 8–10 mg. of Hyflo-Super Cel (Johns-Manville Co. Inc.) is added, and the mixture is stirred with a glass rod. Material on the rod is washed down into the tube with 1 ml. of a 1:4(v/v) ethanol-water mixture, and the tube is then centrifuged for 1 hr. (1500 r.p.m.; radius of centrifuge head, 15 cm.). The supernatant solution is finally sucked from the precipitate with the aid of a fine glass tube attached to a slowly running water pump.

The second and third precipitations are carried out as described above, except that water instead of sodium hydroxide solution is used, and the incubation periods are reduced for convenience to 2 hr. No additional filter aid is added before the centrifugations following the second and third precipitations.

To the final precipitate is added 5 ml. of ethanol, and the pregnanediol is dissolved by warming with stirring at about 75°C. Norite charcoal (*ca.* 1–2 mg.) is then added and the warming continued for 2 min. The

mixture is filtered through a small filter (Whatman No. 1 paper) into a test tube of 2.5 cm. diameter, the centrifuge tube and filter being washed three times with 2-ml. portions of warm ethanol. The filtrate and washings in the tube are evaporated in a water bath under a stream of air, and the residue is finally dried by leaving the tube in a vacuum desiccator over calcium chloride for several hours.

The color reaction is carried out with not more than *ca.* 0.5 mg. of the finally purified product. If, therefore, the amount of the latter appears on inspection to be in excess of 0.5 mg., a suitable aliquot portion is removed after solution in a known volume of ethanol. To the dry pregnanediol 10.0 ml. of concentrated sulfuric acid (A.R.) is added from a buret, and the tube is left in a water bath at 25°C. for 20 min. with occasional shaking. The intensity of the yellow color produced is measured in a Spekker photoelectric absorptiometer using a spectrum violet No. 601 light filter.

The absorptiometer readings are interpreted by reference to a calibration curve made with known amounts of pure pregnane-$3\alpha,20\alpha$-diol varying from 0.1 to 0.5 mg. It is advisable to construct a fresh calibration curve for each batch of unknowns.

Presumably, any suitable photoelectric colorimeter could be used for the final measurement, but care would have to be taken in compiling the calibration curve to ensure that a suitable light filter was employed. Talbot *et al.* state that "the maximum extinction coefficient was obtained with a 420-mμ filter."

Recoveries obtained by Sommerville *et al.* are shown in Table I.

It will be seen that the greater part of the unexplained losses reported by Astwood and Jones and by Talbot *et al.* have been eliminated. Sommerville *et al.* state that their method may be used with some confidence for pregnanediol estimation in urines which contain more than about 2 mg./24-hr. specimen, and this would imply that the method is suitable for a study of the human menstrual cycle. They have also examined the possible interference of certain other steroids in the non-specific sulfuric acid color reaction. Thus androsterone and isoandrosterone, although not completely eliminated by the precipitation process, are less chromogenic than pregnanediol, whereas the powerful chromogens dehydro-*iso*androsterone and pregnane-$3\alpha,17,20$-triol are apparently largely excluded from the final product. Nevertheless the authors do not recommend the method "for pathological urines containing abnormally high concentrations of neutral 17-ketosteroids or of other neutral steroids of adrenal origin."

iii. *Enzymic Hydrolysis.* As mentioned above, the original analysts of free pregnanediol thought that a considerable loss occurred during the hydrolysis of the glucuronide, a view with which, incidentally,

Sommerville *et al.* do not now appear to agree. Accordingly, attempts were made at enzymic hydrolysis. Talbot *et al.* (1943) prepared, from rat liver, a crude enzyme which was capable of hydrolyzing pregnanediol glucuronide. Previously the existence of enzymes capable of hydrolyzing

TABLE I

Recovery of Pregnanediol After the Addition of Sodium Pregnanediol Glucuronidate to Men's Urine

Men's urine specimen	Male urine blank as apparent pregnanediol in $\frac{1}{5}$ of 24-hr. specimen (mg.) (av. of duplicates)	Pregnanediol added as glucuronidate to $\frac{1}{5}$ of 24-hr. urine specimen (mg.)	Pregnanediol recovered (mg.)		Pregnanediol recovery (corrected) (%)
			Apparent	Corrected for blank	
C4	0.016	0.2	0.017	0.001	0
		0.2	0.012	. . . . . .	0
A3	0.008	0.2	0.021	0.013	7
		0.2	0.047	0.039	20
B2	0.024	0.2	0.060	0.036	18
		0.2	0.045	0.021	11
D4	0.035	0.4	0.32	0.29	72
		0.4	0.33	0.29	74
B3	0.015	0.4	0.28	0.27	67
		0.4	0.29	0.28	69
A2	0.018	0.4	0.35	0.33	82
		0.4	0.35	0.33	82
A4	0.044	1.0	0.99	0.95	95
		1.0	0.98	0.93	93
C3	0.019	1.0	0.94	0.92	92
		1.0	0.96	0.94	94
D2	0.077	1.0	1.0	0.92	92
		1.0	0.98	0.90	90
B4	0.030	2.0	1.9	1.9	95
		2.0	2.0	2.0	100
D3	0.017	2.0	2.0	2.0	100
		2.0	1.9	1.9	95
C2	0.026	2.0	1.9	1.9	95
		2.0	1.9	1.9	95

β-glucuronides had been reported by Sera (1914) Masamune (1934), Oshima (1936), Fishman (1939), and Florkin *et al.* (1942). A method of purification of ox spleen glucuronidase was reported by Graham (1946) whilst Fishman and Talalay (1947) have prepared purified extracts from the liver, kidney, and spleen of rats. Talalay *et al.* (1946) and

Mills (1946) have reported studies on the quantitative estimation of β-glucuronidase preparations. However, although the enzymic preparation of Talbot *et al.* (1943) was used by Mason and Kepler (1945) and by Mason and Strickler (1947) for hydrolysis of their urinary glucuronidates (see above) enzymic hydrolysis has not found favor as part of a method for the estimation of urinary pregnanediol.

The method of Astwood and Jones was used by Jones *et al.* (1944) in a study of pregnanediol values during normal pregnancy, and the results may be compared with those obtained on numerous occasions with the Venning method, e.g., Venning and Browne (1937); Stover and Pratt (1939); Cope (1940); Hain (1940, 1942); Smith and Smith (1940); Bachmann *et al.* (1941); Thompson *et al.* (1941); Tien (1941); Venning (1948).

It has been obvious from the time when it became known that pregnanediol was excreted in increased amounts during pregnancy that a pregnanediol determination in urine might be of value as a pregnancy test, e.g., Wilson *et al.* (1939); Hain and Robertson (1939a), Buxton (1940). In 1944, Guterman, using the sulfuric acid color reaction first used for pregnanediol determination by Talbot *et al.* (1941), described a qualitative or very roughly quantitative modification of the Astwood-Talbot technic, which could be rapidly carried out on 100 ml. of urine and which he thought would be of value as a test for pregnancy and as an indication of threatened abortion (which is known to be heralded by a fall in pregnanediol output). Since this test could be performed without the laborious and careful work needed for an accurate assay, it has aroused much interest and has been extensively investigated. The results obtained have been controversial. Thus Guterman (1944, 1945) and McCormack (1946) have claimed an accuracy as great as that of the Friedman test, while Morrow and Benua (1946), Reinhart and Barnes (1946), Bender (1948), Merivale (1948), and Kullander (1948) have not obtained high percentages of accurate diagnoses of pregnancy. In threatened abortion also, Guterman (1946) and Bender (1948) have claimed that the test is of use as an aid to diagnosis and treatment, while Kullander (1948) considers it of no great value.

Among others, Reinhart and Barnes (1946) and Merivale (1948) have recently discussed the value of any pregnanediol estimation as a test for pregnancy. They point out that the values in the luteal phase of the menstrual cycle may overlap those of early pregnancy, and also that the amount of pregnanediol excreted by different women varies within wide limits. It would therefore seem to be very difficult to state values of pregnanediol excretion, even were this accurately determined, above which one might expect to be able to say with a high degree of certainty

that pregnancy had occurred, even when, as Guterman postulated for the success of his test, a period has been missed.

It is clear that a discussion on the exact biological variations of pregnanediol would be pointless in the present state of knowledge, but enough information is available to lend considerable weight to the arguments given above. In addition, Sommerville *et al.* (1948) have criticized the Guterman test on technical grounds.

However, the interest shown in the Guterman method has drawn attention to the need for a method of pregnanediol estimation more easily and quickly carried out than the elaborate procedure given above. Shorter quantitative analytical methods have recently been published, by Guterman and Schroeder (1948) and by Sommerville *et al.* (1948). The two methods are closely similar; however, Guterman and Shroeder finally precipitate pregnanediol from acetone rather than from ethanol as used in the Astwood-Talbot technic.

In their final calculations, Guterman and Schroeder do not state any reliable criteria of purity for the specimens of NaPG which they used for recovery purposes. Their table of pregnanediol recovered from sodium pregnanediol glucuronidate has little relevance to the method. Sommerville *et al.*, on the other hand, added pure *pregnanediol* to urine in their recovery experiments, and this procedure by-passes some of the possible difficulties of the acid hydrolysis.

Guterman and Schroeder point out that there was little interference light transmitted at 430 mμ by the colored products formed with sulfuric acid from androsterone, cholesterol, or the natural estrogens when these substances were added in 0.1 mg. amounts to 100 ml. of urine containing 0.1 mg. pregnanediol, which was then submitted to their process. The shorter procedure of Sommerville *et al.* is given below.

B. RAPID DETERMINATION OF URINARY PREGNANEDIOL (SOMMERVILLE, MARRIAN AND KELLAR, 1948)

Into a 500-ml. flask fitted with an interchangeable ground-glass joint and a reflux condenser is placed 100 ml. of a 24-hr. urine specimen, the volume of which is measured, and 50 ml. of toluene (sulfur-free, redistilled). The contents are heated to boiling and, after the addition of 10 ml. of concentrated hydrochloric acid (A.R. quality), the boiling is continued for exactly 10 min. The mixture is cooled, transferred to a 250-ml. separating funnel, and shaken. After standing for about 5 min., the lower urine layer is run off and discarded, and the upper toluene emulsion layer is filtered through a Buchner funnel with gentle suction, a Whatman No. 1 paper being used to break the emulsion. The filtrate

is then transferred back into the separating funnel, and the lower urine layer that separates from the emulsion is run off and discarded.

The toluene layer is washed in the funnel twice with 15-ml. lots of N NaOH and twice with 15-ml. lots of water. It is then transferred to a 200-ml. round-bottomed flask fitted with a bent ground-glass socket adapter, and evaporated to dryness under reduced pressure in a boiling-water bath.

The residue in the flask is quantitatively transferred with warm ethyl alcohol to a test tube (2.5 cm. diameter) which has a graduation mark at the 5-ml. level. The solution is then evaporated to 5 ml. in a warm water bath under a gentle stream of air. After the tube has been placed in a beaker of water at 75°C., 20 ml. of 0.1 N NaOH is added slowly from a buret over a period of 3 min. and is stirred gently with a glass rod. After a further minute, the beaker and tube are transferred to an incubator at 37°C. and left for 2 hr.

The contents of the tube are then filtered through a sintered glass funnel (3 cm. diameter plate; average pore diameter 20–30 μ), the tube and filter being subsequently washed liberally with water to remove all traces of alkali from the precipitate. The funnel is removed from the filter flask and fitted to a test tube (2.5 cm. diameter) with a side arm. The precipitate is then washed through into the tube with three 5-ml. lots of boiling alcohol under gentle suction.

To the filtrate in the filter tube is added about 1–2 mg. of Norit charcoal, and the mixture is heated in a water bath for 2 min. and filtered through a Whatman No. 1 paper in a conical filter (5 cm. diameter) into a test tube (2.5 cm. diameter). The filtrate is finally evaporated to dryness in a water bath under a stream of air.

To the dry residue in the tube is added 10 ml. of concentrated sulfuric acid (A.R. quality). The tube is placed in a constant-temperature bath at 25°C. and left for 20 min. with occasional shaking. The intensity of the yellow color produced is then measured in a Spekker photoelectric absorptiometer using a spectrum violet No. 601 light filter. The absorptiometer readings are interpreted by reference to a calibration curve made with known amounts of pure pregnane-3α,20α-diol, and the final result is expressed as milligrams of pregnanediol excreted per 24 hr. The calibration curve should be checked at short intervals.

It may be added that Guterman and Schroeder as well as Talbot *et al.* (1941) have shown that any suitable photoelectric colorimeter may be used for measurement of the final color. The maximum extinction appears to be obtained with a light filter at 420–440 mμ. Sommerville *et al.* do not claim "reasonably accurate" analyses at levels of preg-

nanediol excretion below 5 mg. /24 hr.; they warn other workers that the method will be likely to give fictitiously high results when applied to urine containing an abnormally high amount of cholesterol or of neutral 17-ketosteroids, an objection which may not apply so strongly to the technic of Guterman and Schroeder. Sommerville *et al.* only recommend their method "for routine diagnostic purposes" and it is its probable suitability in this respect which justifies its inclusion in the present account.

It seems to the present author that a further gain in the convenience of methods for clinical work might be made by carrying out a creatinine determination on the same urine specimen. With individuals whose creatinine output could be considered constant, it would then be possible to avoid the 24-hr. collection of urines. Pregnanediol output would be expressed as mg./g. of creatinine excreted (Jayle and Libert, 1946, and see above). In this case analyses of "night" specimens of urine could probably be relied on to avoid any very marked difference in the rates of pregnanediol and creatinine excretion.

A promising modification of the methods for determination of free pregnanediol has recently been suggested by Huber (1947). She uses 100 ml. of urine from a 24-hr. specimen, as in the method given above, and the preliminary acid hydrolysis, and removal of acidic substances are carried out also as described. After evaporation of the toluene solutions containing the neutral steroids, the residue is dissolved in benzene and the solution allowed to run through a column of activated aluminum oxide. The pregnanediol is eluted with benzene and then 20% (v/v) benzene/ether. Pregnanediol recovered after evaporation of the solvent is weighed. Good recoveries are reported for amounts of pregnanediol down to 6 mg./l. (0.6 mg./100 ml.) added to urine. However, the number of experiments reported is small, and it is added pregnanediol and not NaPG the recovery of which is reported. A graph is given showing the excretion of pregnanediol found by this method in 5 women, each injected with 100 mg. of progesterone. Huber's method has been used in a study of the menstrual cycle and pregnancy by Zelenka (1948).

Rabinovitch (1948) added zinc dust (1.5 g.) together with concentrated hydrochloric acid (10 ml.) to 100 ml. of urine for hydrolysis. After 5 min. boiling the mixture was cooled and the supernatant decanted on to a sand column, the zinc left in the flask being now washed with dilute hydrochloric acid and water. These washings were added to the sand column which was next dried by a current of hot air. Hot alcohol was used to wash out the original flask and was then poured on to the column. The alcohol which had passed through the column was now

evaporated, and the residue was redissolved in fresh alcohol and precipitated with dilute caustic soda as in the Astwood-Talbot method. The precipitated pregnanediol was collected and estimated by the sulfuric acid color reaction. The outstanding suggestion in this work is the use of zinc dust instead of toluene during the hydrolysis, as a means of preventing possible decomposition of pregnanediol and the formation of interferring colored substances. Recovery figures are not given and it is not possible to assess at present the relative value of the whole method.

An interesting and promising suggestion for a semiquantitative clinical test method has recently been made by Mack and Parks (1947). Using a modified Astwood-Talbot hydrolysis and extraction on 100 ml. of urine, these workers finally estimate roughly the turbidity produced in a final precipitation of pregnanediol from acetone with dilute sodium hydroxide. Such a technic avoids the difficulties of quantitative precipitation and collection of small amounts of precipitate, and also those of a color reaction on an impure product. The reports of other laboratories on this method will be awaited with much interest; in the meantime Mack and Parks consider it of value as a pregnancy test and in threatened abortion. The method might readily be made more accurate, with little extra trouble, by using a stabilizing agent, such as gum ghatti, in preparing the final turbidity and by providing a means of roughly measuring this turbidity and thus relating it to pregnanediol concentration.

Goldzieher (1948) reports an examination of the color produced when pregnanediol reacts with zinc chloride in acetic acid and acetyl chloride. The method has so far been applied to pregnanediol in the purified state.

3. Choice of Method

From what has been said, it may be inferred that there are two distinct trends in the development of methods for "pregnanediol" estimation. The methods of free pregnanediol estimation have become more exact and more specific, so that it might be said that here the final perfect analysis is at least in sight.

On the other hand recognition of the apparent impossibility of separating, by simple means, the mixture of steroid glucuronides in human urine has led to a broadening of the scope of the combined pregnanediol methods to include steroid glucuronides or pregnanediol-like glucuronide with, at present, an uncertain degree of specificity. The situation here may be likened to that existing when an analysis of 17-ketosteroids is made; a knowledge of the concentration of a group of substances rather than of a single compound is the object of the estimation. It must be added that it is not yet known with what success this object has been

attained with combined pregnanediol methods; this uncertainty does not of course now exist with 17-ketosteroid analyses.

Choice of method will depend on the kind of result needed. If a knowledge of the output of pregnanediol itself is required with the greatest possible accuracy, the method of Sommerville *et al.*, given above, may be recommended; if less accuracy, for less work, is needed, the shorter method, due to Sommerville *et al.* may suffice, see e.g., Swyer (1949). For larger amounts of free pregnanediol, the modification of Huber (1947) may save much labor. For clinical work, it is to be hoped that the method of Mack and Parks (1947) will receive attention.

If it is desired to investigate the menstrual cycle, one of the (perhaps) easier and quicker methods of Jayle and Libert (1946) or Bisset *et al.* (1948) for estimation of a definite glucuronide fraction may be preferred, especially since such a fraction will probably include metabolites of progesterone, such as pregnane 3α-ol-20-one, which are not determined by a free pregnanediol procedure. The clinical value of such methods remains to be determined.

For pregnanediol estimations in late pregnancy, Allen and Viergiver's (1941) technic will probably give quite accurate results, since the extra glucuronide estimated by this method will not be more than a small percentage of the total (cf. Lyon, 1946).

For preparative purposes and, perhaps, too, for the estimation of a fraction rich in progesterone metabolites in high concentrations in urine, the original method of Venning is likely to remain of permanent value.

4. Some Results

Most of the results reported in the literature were obtained by the Venning method and are therefore open to the criticisms mentioned above. The range found for so-called normal values is extremely wide, and it is doubtful whether a collection and statistical expression of the reported values would be of help to other workers at present. It is to be hoped, however, that such an analysis will eventually be made on a large collection of results obtained with methods of known and definable accuracy. An indication of values may, however, be given here.

Thus, all workers with the Venning method are agreed that there is little or no pregnanediol excreted in the follicular phase of the human menstrual cycle. In the luteal phase, Venning and Browne (1937) give 45–55 mg. (as pregnanediol) as the range of total amount excreted in the luteal phase, but Pattee *et al.* (1940) cite a patient who excreted 112, 96, and 286 mg. of pregnanediol in 3 successive cycles. Wilson *et al.* (1939) give, amongst menstrual cycle values (5 cases) an average peak of about 3.5 mg./24 hr. excreted on the seventh day premenstrual; the correspond-

ing value of Venning and Browne (1938) is about 4.5 mg./24 hr. on the sixth day premenstrual (10 cases).   Wooster (1942) in a review article, quoted 3-50 mg. as the total quantity excreted in a menstrual cycle.

Kaufmann and Westphal (1947) have reported pregnanediol figures, obtained with Westphal's (1944) barium pregnanediol glucuronidate method (see above), for thirteen normal non-pregnant women.   Excre-
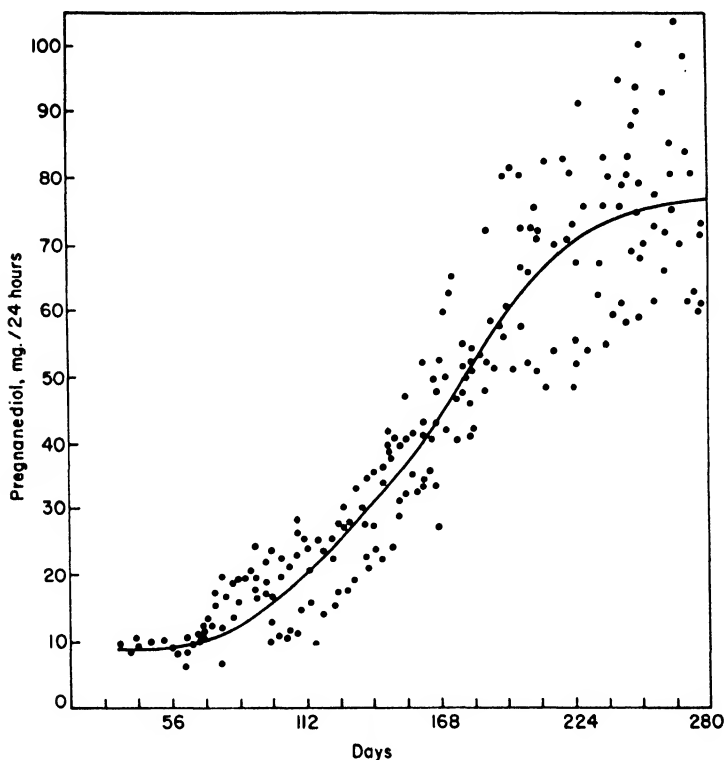
FIG. 3.   Pregnanediol in eight cases of normal pregnancy (Venning, 1938).

tion of pregnanediol was found to last 8-14 days of the menstrual cycle and to vary from 5-24 mg./24 hr.

In pregnancy, Venning (1938) gives Fig. 3 and Wilson and Randall (1938), Fig. 4.   Other menstrual cycle results are given by Hamblen et al. (1939), Hain and Robertson (1939b), Stover and Pratt (1939), Cope (1940), and many other workers, while in pregnancy more figures are quoted by Wilson and Randall (1938, 1939), Smith and Smith (1940), Hain (1940, 1942), Bachman et al. (1941), amongst many others, all using the Venning method.   See also Venning (1948).

The method of Astwood and Jones (1941) was used by Jones et al.

(1944) in a study of pregnancy.   Results at the onset of labor are given
by Lyon (1946) with the Allen and Viergiver (1941) method.   From a
study of more than 100 cases, Lyon concludes "the onset of labor is
preceded for several days by a marked decline in the excretion of urinary
sodium pregnanediol glucuronidate.   These declines are greater than
any previously recorded. . . . It is implied that the concentration of
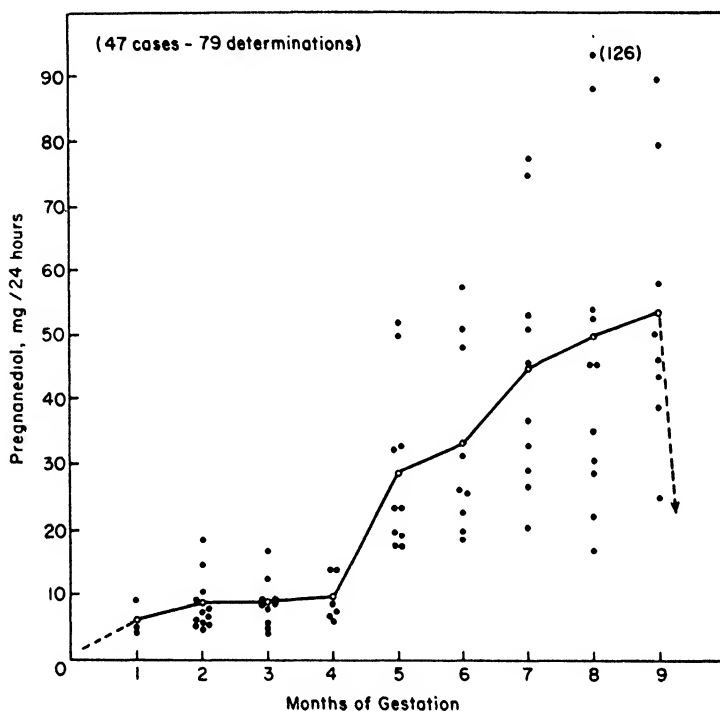
FIG. 4.   Excretion of pregnanediol in the urine of pregnant women (Method of
Venning, 1938).   (From Wilson and Randall, *Proc. Staff Meetings Mayo Clinic* **13**,
813, 1938.)

progesterone available at the onset of labor is insufficient to maintain and
continue pregnancy."

The output of pregnanediol by man after administered progesterone
has been studied (Venning method) by Stover and Pratt (1939), Buxton
and Westphal (1939), Hamblen *et al.* (1939), Hamblen *et al.* (1940),
Venning and Browne (1940), Westphal (1942), and Davis and Fugo
(1947).

Allen *et al.* (1944) give figures obtained by the method of Allen and
Viergiver (1941) for pregnanediol excreted by patients, with second-

ary amenorrhea, who had received orally doses of progesterone and anhydroxyprogesterone.

Pregnanediol presumably formed from administered deoxycorticosterone has been estimated by, e.g., Cuyler *et al.* (1940), Westphal (1942), and Horwitt *et al.* (1944).

In adrenal tumors and hyperplasia pregnanediol figures are increased, e.g., Venning *et al.* (1938), Finkler (1941), Salmon *et al.* (1941), Genitis and Bronstein (1942), Talbot *et al.* (1942), Anderson *et al.* (1943), but it is perhaps wiser to reserve judgment on the actual values obtained until more is known about possible interference in the methods used by steroids other than pregnanediol known to be excreted in increased amounts in such cases.

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CHAPTER XIX

Thyroidal Substances

BY E. P. REINEKE AND C. W. TURNER

CONTENTS

I. INTRODUCTION

Because of its varied and diverse actions in regulating body processes a considerable number of functions have been suggested as a basis for the assay of thyroid hormone. These can be grouped into four main categories as follows:

1. Assays based on the effect of thyroid in altering the energy metabolism or body weight.
2. Replacement therapy in thyroidectomized or thiouracil-treated animals.
3. Maintenance of the thyroid-pituitary balance in thiouracil-treated animals.
4. Stimulation of processes of maturation and metamorphosis in amphibian tadpoles.

The choice of an assay for a particular purpose will depend on a variety of factors such as the specificity and precision required, the laboratory facilities and animals available, and the nature of the information desired. The methods now available are rather laborious and time-consuming, particularly if assays are desired on a large number of preparations. If suitable apparatus is available and only a limited number of samples are to be compared, one of the metabolic methods employing mice or guinea pigs would probably be the method of choice. The number of assays that can be conducted, however, will be limited by the time involved in the metabolism determinations. The assays based on thyroid weight measurements in thiouracil-treated animals as well as the tadpole methods usually permit a larger number of comparisons to be made and also require little special equipment for their use. In all thyroid assays rigid control of environmental conditions is desirable since variations in temperature or nutrition of the animals will have a pronounced effect on the response. It is also necessary to employ a suitable reference standard since the animal variability is too great from time to time and from one laboratory to another to permit defining assay units in terms of a given animal response.

II. Thyroid Standards and Routes of Administration

1. Thyroxine

As crystalline thyroxine of good purity is now readily available it would serve as a product of uniform potency for use as a primary standard in thyroid assays. However, a number of points still remain to be clarified before assays of the comparative potency of thyroxine and thyroidal proteins can be satisfactorily interpreted. The thyroxine obtained by organic synthesis or by isolation after alkaline hydrolysis of thyroxine-containing proteins is a racemic mixture composed of the L- and D-isomers. Both thyroid (Harington and Salter, 1930) and synthetic thyroprotein (Reineke and Turner, 1943) contain pure L-thyroxine. It is a question of considerable importance, therefore, whether

a part of the physiological activity of a racemic mixture resides in the natural L-component or whether D-thyroxine also possesses some activity. Both D- and L-thyroxine that were separated by Harington (1928) by resolution from a racemic mixture were assayed by Gaddum who reported that in frog tadpoles (1927–28) and in rats (1929–30) the D-isomer showed about one-third the potency of the L-form. The same compounds appeared to have equal activity in myxedema patients (Salter, Lerman, and Means, 1935). L-Thyroxine isolated from an enzyme hydrolysate of thyroid substance was reported by Foster *et al.* (1936) to exert twice the metabolic effect of a racemic mixture. Similarly, L-thyroxine isolated from an acid hydrolysate of iodinated casein produced twice the effect of racemic thyroxine in guinea pigs (Reineke and Turner, 1943) and tadpoles, chicks, and rats (Reineke and Turner, 1945a). The latter investigations indicate that all the activity of D,L-thyroxine can be accounted for by the L-isomer, and infer, therefore, that the D-form has little or no activity. More recently, however, Deanesly and Parkes (1945a) have reported no difference in potency of L- and D,L-thyroxine when assayed in *Xenopus* tadpoles. By use of a method based on the prevention of changes in the basophilic cells in the pituitary of hypothyroid rats L-thyroxine obtained from three different sources was found to have only 1.5 times the potency of the racemic form (Griesbach, Kennedy, and Purves, 1947).

The preparation of D-thyroxine has been described by Pitt-Rivers and Lerman (1948). Its physiological activity in myxedematous patients was found to be between one-eighth and one-tenth that of L-thyroxine.

It seems possible that these apparent discrepancies may be due at least in part to a differential action of the isomers on the particular function taken as an end point for the assay. No investigations bearing specifically on this point have been reported, however. In view of the consistent results obtained in their investigations in several species the authors have assumed an activity ratio of L- to D,L-thyroxine of 2:1 in attempting to correlate biological assay results with the thyroxine content of iodinated proteins.

2. Thyroid and Thyroprotein

Although thyroxine, natural thyroid substance, and thyroactive iodinated proteins are all effective when administered either orally or parenterally, their relative effectiveness may vary considerably, depending upon the completeness of absorption of the particular substance. Thompson *et al.* (1939) estimated that when given orally in *man* free thyroxine is only one thirty-third as effective as the monosodium salt or

one-ninetieth as effective as the disodium salt. Data reported by Dressler and Holling (1940) indicate that thyroxine given orally to guinea pigs as the sodium salt is utilized only 42% as effectively as by subcutaneous injection. Monroe and Turner (1949) have obtained similar results in the chick. In guinea pigs (Reineke *et al.*, 1945) iodinated proteins were much more effective when injected intraperitoneally than by the subcutaneous route. In sheep (Turner and Reineke, 1946) thyroxine was only one-eighth as effective orally as by injection; iodinated proteins showed only 5% of the effect orally as by subcutaneous injection. Similar comparisons in rats (Frieden and Winzler, 1948) indicate that iodinated protein given orally produces 15% of the effect observed by intraperitoneal injection. The problem is complicated further by the fact that many investigators have reported greater potency in thyroid substance than can be accounted for by its thyroxine content. This has led to the suggestion that a particular linkage of thyroxine in thyroid protein may enhance its activity (see discussions by Kendall, 1929; Harington, 1933; Means, 1937; Elmer, 1938; Salter, 1940). Frieden and Winzler (1948) reported that when both thyroxine and natural thyroid proteins were given parenterally the latter consistently showed more activity than expected from their thyroxine content, even assuming that L-thyroxine has twice the potency of the racemic mixture used for comparison. The same workers found that synthetic thyroprotein was less active biologically than its L-thyroxine content would indicate. However, they admitted that quite possibly their estimates of thyroxine content were too high, suggesting the presence of some inactive compound or compounds in the butanol-soluble fraction of thyroprotein hydrolysate. In our studies synthetic thyroproteins show good agreement between the results of chemical analyses for thyroxine-like iodine and biological assay values determined by their metabolic action in guinea pigs (Reineke *et al.*, 1945). However, preliminary determinations of the thyroxine content of such preparations by a new isotope dilution technic employing radioactive thyroxine as a standard (Reineke *et al.*, 1949) show that the values obtained by the previous method are considerably too high. Consequently the observed thyroidal potency of such preparations when given parenterally is also greater than would be expected from their true thyroxine content. From the above considerations, it is clear that standards used as a reference point in thyroid assays should be as similar in all respects to the substance being tested as possible. Furthermore, the same method of administration should be employed for both the standard and the test substance if direct comparisons are to be made. These requirements can be met most easily by setting aside as a laboratory standard a preparation of the type being

investigated and determining as completely as possible its thyroidal potency and thyroxine content. This will then serve as a reference compound for further work.

III. Assays Based on Elevation of the Metabolic Rate

1. Clinical Assays

The discovery o Magnus-Levy (1895) that thyroid administration causes an increase in the oxygen-carbon dioxide exchange provided the basis for modern clinical metabolism determinations as one measure of the thyroid status. In fact, hypothyroid patients have been employed successfully for the assay of thyroidal substances (Salter *et al.*, 1933; Means *et al.*, 1933; Lerman and Salter, 1934a, b). It is stated that once a given patient's response to a standard preparation has been established, test preparations can be assayed with a precision of approximately $\pm 15\%$ (Salter, 1940).

2. Metabolism of Laboratory Animals

For determinations of metabolism in laboratory animals a suitable respirometer will be required. This may consist either of the open circuit type, most suitable for the measurement of CO_2 production (Mørch, 1929) or the closed circuit type which permits direct measurement of the volume of O_2 consumed (Teitelbaum and Horne, 1941). Details of the construction and operation of both general types of apparatus may be found in the excellent book by Brody (1945). Numerous modifications of both types have been devised.

A. RAT ASSAYS

Gaddum (1929) determined the oxygen consumption of rats before and after the administration of thyroxine and several related compounds as a measure of their comparative potency. No advantage was found in the use of thyroidectomized rats. However, Meyer and Wertz (1939) reported thyroidectomized rats to be 25 to 30 times as sensitive as normal rats in their response to thyroidal stimulation. Metabolism determinations were made under strong lights after a 20-hr. fast. After establishing basal values on the test rats thyroid medication was given by stomach tube for 3 consecutive days, and the elevation in metabolism was observed on the fifth day. An increase of 25 to 30% in O_2 consumption was considered to be most suitable for comparisons. A period of 4 to 8 weeks was allowed to elapse after thyroidectomy before the rats were used for assays. At this time the oxygen consumption had declined from the normal of 1400–1600 mg. down to 900–1200 mg./hr./kg. Although

thyroidectomized rats appear to offer satisfactory sensitivity for thyroid assays, the waiting period required to reach the hypothyroid condition definitely restricts the utility of this method.

B. MOUSE METABOLISM ASSAY

An extremely precise method of assay which is stated to yield results within $\pm 15\%$ in individual mice has been reported by Mørch (1929). Male mice weighing 16–22 g. are placed on a standard diet consisting of 4 g. of dry food and 3 ml. of skim milk daily per 20 g. body weight for a period of 8 days. The normal metabolism is determined in 5 consecutive 24-hr. periods with the mouse and its daily food allotment placed in an open circuit metabolism chamber maintained at exactly 23°C. Thyroid substance is then mixed with the food supply in an amount to supply from 0.1 to 1.0 g. per 20 g. body weight. If the mice gain or lose weight, the dosage is adjusted so that the daily dose per 20 g. remains constant. After 3 weeks, the CO_2 output is determined as before during five 24-hr. periods. If the animals are losing weight, it is recommended that the mean CO_2 output per gram of body weight be computed. If they are gaining weight the CO_2 output is expressed in milligrams per square centimeter surface area. Surface area of the mouse is determined by the formula:

$$\text{Area in cm.}^2 = 11.36 \times \text{grams of weight}^{\frac{2}{3}}$$

In using this method it is necessary to establish a dose-response curve by administering graded amounts of the standard under the exact experimental conditions to be employed in the assays. The activity of test preparations in terms of the standard can then be determined by reference to the amount which when given by mouth daily for 3 weeks and expressed as milligrams daily per kilogram mouse is able to elicit a 15% increase in CO_2 output.

C. GUINEA PIG METABOLISM ASSAY

The guinea pig is a very suitable animal for thyroid assays in that it is highly sensitive and shows a satisfactory relationship between the thyroid dosage and the percentage increase in metabolism. Dressler and Holling (1940) recommended that feed and water be withheld for 18 hr. before determination of the O_2 consumption and CO_2 output, the metabolism being expressed as milliliters of O_2 or CO_2 exchanged per 100 g. body weight per hour. After control readings had been taken on 3 separate days, the thyroid preparations were administered orally for 4 consecutive days. One hundred hours after the first dosage a BMR determination was made as before, and the percentage increase was

calculated for each animal against its previously established control value. A reference curve was established by graded dosage with a standard thyroid powder. The potency of test preparations could then be computed in terms of the standard. A metabolism unit was defined as 1 mg. of standard powder, or that amount which when supplied daily per 100 g. body weight to 300–500 g. guinea pigs, the metabolism being measured 100 hr. after the first dosage, will increase the O_2 consumption
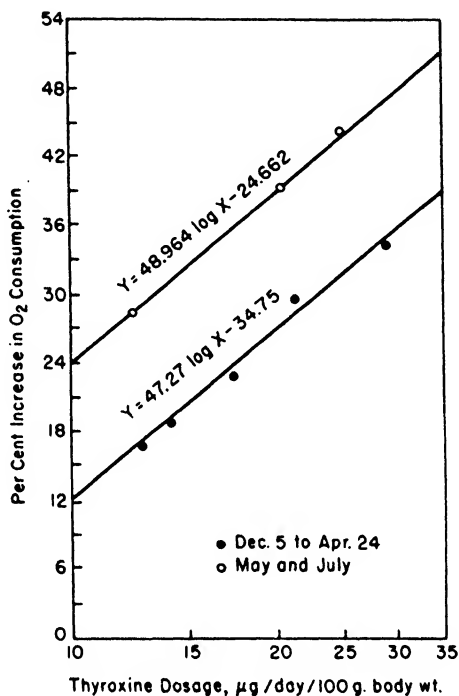
FIG. 1. The relation between thyroxine dosage and the percentage increase in oxygen consumption of groups of normal, partially fasted guinea pigs. (From Reineke and Turner 1942.)

31% and the CO_2 output 29%. The mean error for single assays with 10 animals in a group was estimated to be $\pm 17\%$. A similar method has been described by Reineke and Turner (1942). Male guinea pigs weighing 200–300 g. were trained to eat their daily feed allotment during the afternoon and all feed was removed from their cages at about 10:00 P.M. in order to permit daily measurements of O_2 consumption in a partially fasted condition. Under these conditions it was found that the oxygen consumption of normal guinea pigs housed under uniform environmental conditions could be predicted from their body weight

within the range of -2.5 to $+4.3\%$, thus eliminating the necessity of determining the normal metabolism in each assay. The metabolic effect of a test substance could then be determined by measuring the average oxygen consumption on the fourth and fifth days of dosage and computing the percentage deviation from the predicted value for an animal of the same weight. During the season of warm weather there was a decline of about 15% in the normal metabolism values and also an increase in sensitivity to thyroxine stimulation. In either case the percentage increase in oxygen consumption on a graded series of thyroxine dosages given orally varied directly with the logarithm of the dosage (Fig. 1), the slopes of the curves being similar. Having established the relation between the dosage of thyroxine and the percentage increase in O_2 consumption, one can calculate the thyroxine equivalent of test preparations. By substituting the percentage increase in oxygen consumption for y in the appropriate equation (Fig. 1) and solving for x, one obtains the logarithm of the thyroxine dosage that must be given orally to produce the same response. By looking up the antilogarithm, the actual thyroxine equivalent is obtained. As pointed out previously, in the use of this type of assay the reference curve should be established for a standard substance similar in so far as possible to those being tested.

3. Asphyxiation Method

Rats treated with thyroid show increased sensitivity to an O_2 deficiency (Duran, 1920). Conversely, Gordon et al. reported an increased resistance to lowered barometric pressures in rats receiving thiourea (1944a), dilantin, and thiouracil (1944b). Smith et al. (1947a) reported that the survival time of mice held in sealed 1-qt. jars was decreased by treatment with iodinated casein, and the relationship between log dose and survival time was linear. Survival times after 1, 2, and 3 weeks of treatment were not significantly different; males were less variable than females in their response. Elevation of the environmental temperature increased, while lowering of the temperature decreased the response. These authors state that, using survival time at 23°C. in 1-qt. jars, an assay employing forty 16- to 20-g. male mice per test substance should give a result within 80–124% of the true value in 19 out of 20 tests. It was reported by the same authors (1947b) that death of normal mice occurred at concentrations of 4% O_2 and 14% CO_2. In hyperthyroid mice death occurred with 8% O_2 and 10% CO_2 in the chamber, indicating that death occurs earlier in hyperthyroid animals due to increased susceptibility to anoxia. Very similar results were reported by Hutcheon (1948). Adult mice weighing from 20 to 25 g. were divided into groups of 10 animals, including a control group and 3 groups which

received injections of 2.5, 5.0, or 10 μg. of thyroxine daily for 7 days. The mice were all placed together in a 32-l. airtight chamber containing soda lime for about 45 min., or until approximately half of them had died. It was found that the percentage of mortality varied linearly as the logarithm of the dosage. Because of its extreme simplicity this method may prove to be very useful.

IV. Assays Based on Loss in Body Weight

The well-known ability of thyroid in high dosage to reduce body weight can be used as a simple and fairly satisfactory assay, providing that a sufficiently responsive species of animal is selected and the daily food intake is restricted to a constant amount.

1. Weight Reduction in Guinea Pigs

As recommended by Kreitmair (1928), groups of 4 guinea pigs weighing 250-300 g. are weighed in a partially fasted condition on Monday morning, and then daily dosage with the test preparation is begun at levels of 0.001, 0.01, and 0.1 g. The feed is so measured that it is all eaten during the day and the following night. The animals receive their sixth and last dose on Saturday forenoon, and final body weights are recorded on the following Monday morning. A dose is considered effective when it causes a weight loss of 10% in at least 3 out of 4 animals. It is suggested that assay values be expressed as guinea pig units per gram of substance by dividing the daily dose in milligrams into 1000. As judged by the response to graded doses of thyroxine (Reineke and Turner, 1942) groups of 4 animals at the 10% weight reduction level will show an assay value within ± 25% of the true value in approximately two-thirds of the cases.

2. Weight Reduction in Sheep

With the development of methods for the formation of synthetic thyroproteins suitable for use in domestic animals (see review by Reineke, 1946), it became a matter of considerable importance to measure the utilization of such preparations in ruminant animals. For this purpose, Turner and Reineke (1946) employed an assay method based on the weight-reducing properties of such substances in sheep. Lots of 4 sheep of uniform type and weight were used. They were fed *ad libitum*, but water was withheld for approximately 20 hr. before weighing. Body weights were taken on 3 successive days at the beginning and again during the last 3 days of the 14-day assay period, the thyroprotein being administered once daily in a gelatin capsule. The average percentage decline in body weight of the group of 4 animals during the 2-week period was

used as the index of biological activity of the preparation. Although the statistical limits of error of this method have not been computed, there was fair agreement between the thyroxine content of various preparations as determined by a chemical method and their biological activity in sheep.

3. Transitory Weight Loss in Rats

Hutcheon (1948) reported that no significant difference between the weight losses of normal and thyroid-treated rats could be found after a 24-hr. starvation period. Rats allowed free access to food and water, however, showed significant decreases in body weight during the 48-hr. period following the first injection of thyroxine. Male rats weighing 220–280 g. were given 0.1% thiouracil in their drinking water for 10 days prior to thyroid treatment. When thyroxine was then injected subcutaneously on 2 successive days in amounts of 0.25–2.0 mg., the weight loss in 48 hr. varied linearly in proportion to the logarithm of the dose. However, an assay of this type would require 400 animals (200 on the standard and 200 on the unknown) for limits of $\pm 25\%$ at $P = 0.95$.

V. Growth Restoration in Thyroidectomized Animals

It has been observed by many workers that subsequent to thyroidectomy in young animals, growth stasis occurs accompanied by subjective symptoms of hypothyroidism. Pick and Pineles (1909) used replacement therapy in thyroidectomized goats to test the activity of thyroid preparations. In early tests on synthetic thyroproteins, Reineke and Turner (1941) also used young thyroidectomized goats as a test of specific thyroidal activity. Young goats thyroidectomized when less than a month old reach a complete growth stasis 1 to 2 months after the operation. Thyroprotein, given orally at several dosage levels, caused growth stimulation that was roughly proportional to the dosage. Rowlands (1945) investigated the possibility of employing replacement therapy in thyroidectomized rats as a quantitative assay for iodinated proteins. Although growth could be restored by either dried thyroid or iodinated Ardein the dose-response curve was so flat that huge numbers of rats would have to be injected at each level of dosage to make the differences in response statistically significant. It was calculated that an assay comparing a test sample against a standard would require 6100 animals for an error of $\pm 10\%$ at $P = 0.95$. Obviously this method would be out of the question as a quantitative assay. However, growth restoration in thyroidectomized animals can be used to good advantage as a qualitative test in some cases since it is highly specific for thyroid hormone.

VI. MAINTENANCE OF THE THYROID-PITUITARY BALANCE IN THIOURACIL-TREATED ANIMALS

1. The Goiter Prevention Method

The discovery of a series of goitrogenic compounds (Kennedy and Purves, 1941; Kennedy, 1942; Mackenzie *et al.*, 1941; Richter and Clisby, 1942) has made possible a new type of thyroid assay based on maintenance of the hormone balance between the thyroid and pituitary glands. It was established by the studies of Mackenzie and Mackenzie (1943), Astwood *et al.* (1943), Purves (1943) and Griesbach and Purves (1943) that the goitrogenic action is due to inhibition of thyroid hormone formation, which in turn permits increased secretion of thyrotropic hormone by the pituitary and compensatory hypertrophy of the thyroid. Thyroid enlargement can be prevented by administering thyroxine or thyroid hormone in sufficient amounts to bring the pituitary and thyroid hormone back into normal balance. Dempsey and Astwood (1943) first showed that the depression of the thyroid weight in thiouracil-treated rats bore a quantitative relation to the thyroxine dosage, and used this response to determine the thyroid secretion rate at several different environmental temperatures. Mixner *et al.* (1944) found that the thyroid of the chick responded in a similar manner and reported the effect of a number of variables on the response. Maximum thyroid enlargement was observed with 0.1% of thiouracil in either the feed or water. An assay period of 10 to 14 days was recommended for thyroid assays or the measurement of thyroid secretion rates. Female chicks consistently showed a higher thyroid secretion rate than males. Thyroid assays determined by this method in rats (Reineke *et al.* 1945) coincided closely with results obtained by a metabolic method (Fig. 2). The curve relating thyroid weight to thyroxine dosage is a straight line and could be used as a reference curve for test preparations run under similar conditions. The intercept of the response curve with the normal thyroid weight can be taken as a measure of the thyroid secretion rate since it represents the amount of D,L-thyroxine required to maintain the normal thyroid-pituitary balance. Since the rats used in this trial averaged approximately 140 g. the thyroid secretion rate per 100 g. body weight was 3.47 μg. Although this procedure is known to yield quite consistent values, the statistical limits of error have not been determined. As usually employed, male rats weighing 100–200 g. are apportioned as evenly as possible into groups of 8–10 animals. Five groups are usually sufficient to establish a response curve. During the period of treatment all groups are given 0.1% thiouracil in their feed. Four groups are

injected with thyroxine daily in graduated dosages of 1–4 μg./100 g. body weight. If the thyroid secretion rate is to be determined a group of normal rats not receiving thiouracil or thyroxine is included. Dosages of test preparations are given in amounts calculated as nearly as possible to fall in the middle of the response curve. After 14 days the rats are sacrificed, and their thyroids are dissected out cleanly on moist filter
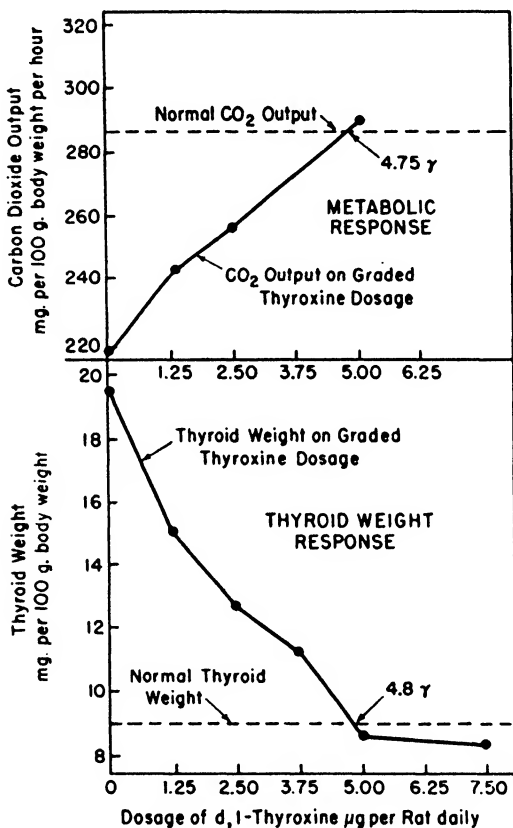
FIG. 2. Influence of graded doses of thyroxine on the metabolism and thyroid weight of thiouracil-treated rats. (From Reineke, Mixner, and Turner, 1945.)

paper and weighed rapidly to avoid evaporation losses. Thyroid weights are reported as milligrams per 100 g. body weight. If chicks are used, at least 20 animals are required per group since the response is somewhat more variable than in the rat. Otherwise the assay procedure is similar. In either case the nutrition and environmental temperature should be kept as uniform as possible. It is not certain that the amount of hormone required to maintain the thyroid-pituitary balance would be the

same if supplied by daily injections as when secreted by the thyroid gland. In the first instance, additions to the circulating hormone are intermittent; in the gland, itself, hormone secretion and discharge appears to be a continuous process. Nevertheless the results obtained by the method are quite consistent and provide valuable information on the thyroid state. As pointed out by Hurst and Turner (1948), thyroid secretion studies provide a valuable landmark in determination of the "physiological" dosage range in studying the effects of thyroidal stimulation on the various processes of laboratory and domestic animals.

2. Restoration of Thyroidectomy Changes in the Pituitary

In 1941 Griesbach described marked changes in the basophil cells of the anterior pituitary of rats with hyperplastic thyroids. In further investigations (Griesbach and Purves, 1943) it was found that severe thyroxine deficiency causes almost complete degranulation of acidophil cells and an increase in basophils. Purves and Griesbach (1946) conclude that the basophil cells are the site of thyrotropic hormone secretion. They report that even in a slightly hypothyroid animal the basophils increase in size, while the acidophils become degranulated only in complete thyroid deficiency. One-half microgram of D,L-thyroxine daily per 100 g. body weight was sufficient to maintain normal acidophil granules, but 2.25 μg. were required to prevent basophil changes, this value being considered to represent the thyroid secretion rate. The condition of the pituitary basophils appears to be a very sensitive indicator of the thyroid state. As cited earlier, Griesbach et al. (1947) used the suppression of basophil changes in thyroidectomized rats as an end point in comparing the activity of L- and D,L-thyroxine. The value for the thyroid secretion rate of 2.25 μg. D,L-thyroxine obtained by this method is considerably lower than that obtained by all investigators using the goiter prevention method. Whether this represents a difference in the strains of rats used or some as yet unexplained factor entering into the dynamics of the thyroid-pituitary balance is not clear at this time.

3. Comparative Studies on the Thyroid Secretion Rate

Since the development of a suitable technic for its measurement, the thyroid secretion rate has been determined in several species of animals under a variety of conditions. Dempsey and Astwood (1943) demonstrated that the thyroid secretion rate of rats is increased markedly at low environmental temperatures and depressed at high temperatures. A seasonal trend in the thyroid secretion rate of young chicks was observed (Reineke and Turner, 1945b), the rate in spring and summer being only about one-half the winter level. Mixner and Upp (1947)

TABLE I

Thyroid Secretion Rate of Several Species

Animal	Body weight	Thyroid secretion rate as μg. D,L-thyroxine/day μg.	Daily thyroid secretion rate as μg. D,L-thyroxine/100 g. body weight μg.	Temperature
White Leghorn cockerels[a]	343.0 g.	7.55	2.20	82°–86°F.
⸲	1536.0 g.	25.00	1.36	82°–86°
White Plymouth Rock cockerels[a]	410.0 g.	8.10	1.98	82°–86°
	1502.0 g.	23.00	1.53	82°–86°
White Plymouth Rock pullets[a]	360.0 g.	8.75	2.43	82°–86°
	1637.0 g.	26.00	1.59	82°–86°
White Leghorn hens[d] (2-yr.-old)	1900.0 g.	12.00	0.6	
White Pekin ducks males[e]	488.0 g.	13.9	2.85	78°F.
	1691.0 g.	43.2	2.55	
White Pekin ducks females[e]	524.0 g.	14.0	2.67	78°
	1617.0 g.	48.0	2.97	
Growing goats[a]				
Males and females	10.0 kg.	180.00	1.80	50°-80° probable range
Females	20.4 kg.	640.00	3.13	50°–80° probable range
Females	34.5 kg.	930.00	2.70	50°-80° probable range
Growing male rats[b]	85.2 g.	3.10	3.64	78°
	126.9 g.	4.18	3.29	78°
	168.7 g.	5.90	3.50	78°
	219.5 g.	7.46	3.40	78°
	275.7 g.	9.54	3.46	78°
Growing female rats[b]	81.1 g.	3.75	4.63	78°
	128.1 g.	5.19	4.05	78°
	171.9 g.	5.74	3.34	78°
	223.6 g.	6.53	2.92	78°
	266.2 g.	7.51	2.82	78°
Pregnant rats[b]	190.8 g.	5.59	2.93	78°
Lactating rats[b]	209.1 g.	6.48	3.10	78°
Mature male mice[c]				
Schwing strain	22.4 g.	0.50	2.4	80°
Schwing strain	23.6 g.	0.17	0.7	87°
Rockland strain	36.7 g.	1.00	2.8	80°
Mature female mice[c]				
Schwing strain	19.9 g.	1.10	5.5	80°
Schwing strain	21.4 g.	0.68	3.2	87°
Rockland strain	32.7 g.	0.70	2.1	80°
C₂H strain	19.6 g.	0.40	2.2	80°

[a] Schultze and Turner (1945).   [c] Hurst and Turner (1948).   [e] Biellier and Turner (1950).
[b] Monroe and Turner (1946).   [d] Turner (1948).

reported that "double cross" hybrid chicks obtained by crossing four closely inbred lines had a considerably higher thyroid secretion rate than either "single cross" chicks or ordinary breeds. They suggest that the increased thyroid function may be a factor in heterosis or so-called hybrid vigor. Data regarding the thyroid secretion rates of several species are compiled in Table I. Female chicks consistently show a higher secretion rate than males, and in both sexes there is a decline in the hormone secretion per unit body weight as the animal grows larger. There is a pronounced decline in the secretion rate of older hens. In male rats the secretion rate per unit body weight is nearly constant, whereas in females the rate declines with increasing size. Rather surprisingly, no significant differences from the normal were observed in pregnant or lactating rats. The effect of environmental temperature on the thyroid mechanism is illustrated by the pronounced decline in thyroid secretion rates of mice when the temperature was increased from 80° to 87°F. Some interesting differences appear in the thyroxine secretion rates of the three strains of mice that are compared. In the Schwing strain, females appear to secrete more thyroxine than males, whereas in the Rockland strain the situation is reversed. When expressed as micrograms D,L-thyroxine per 100 g. body weight the thyroxine secretion rate of the five species represented falls within the range of 0.6–5.5. During growth, at least in chicks and rats, the thyroid secretion increases at a constantly decreasing rate and can be expressed mathematically by an equation of the form: $y = ax^b$, in which y is the secretion rate in micrograms D,L-thyroxine, a is a constant, x is body weight, and b is an exponent derived from the rate of increase in thyroxine secretion with increasing body weight. It will be noted that the exponent b (Table II) for thyroid secretion rate is less than 1 in all cases. This

TABLE II

Equations Relating the Thyroid Secretion Rate and Thyroid Weight to Body Weight

Animals	y = Thyroid secretion, μg. D,L-thyroxine x = Body wt., g.	y = Thyroid wt., mg. x = Body wt., g.
Growing Chickens		
White Leghorn, male[a]	$y = 0.065x^{0.81}$	$y = 0.158x^{0.88}$
White Plymouth Rock, male[a]	$y = 0.053x^{0.83}$	$y = 0.035x^{1.05}$
White Plymouth Rock, female[a]	$y = 0.073x^{0.80}$	$y = 0.062x^{0.99}$
Growing Rats		
Male[b]	$y = 0.041x^{0.97}$	$y = 0.136x^{0.90}$
Female[b]	$y = 0.422x^{0.61}$	$y = 0.230x^{0.81}$

[a] Schultze and Turner (1945).
[b] Monroe and Turner (1946).

means that the thyroid secretion increases at a slower rate than does body size. In all except one instance (male rats), the thyroid weight is shown to increase somewhat more rapidly than the thyroid secretion rate. Since many factors other than body size have been shown to influence thyroid function, these equations would only be applicable under conditions similar to those in which the experiments were conducted.

VII. Assays Based on Stimulation of Metamorphosis in Amphibian Tadpoles

1. Function of the Thyroid in Metamorphosis

The induction of premature metamorphosis in amphibian tadpoles by administering thyroid substance (Gudernatsch, 1913a, b) has been studied by numerous workers with the view to adapting this response to the assay of thyroidal materials. Romeis (1915) noted that the precocious differentiation of the body was accompanied by a decrease in weight due to increased metabolism and loss of water. Thyroidectomy of tadpoles at the 5- to 6-mm. stage prevented metamorphosis, and such animals grew to a large size in the larval stage (Allen, 1916, 1918; Hoskins and Morris, 1917, 1919; Terry, 1918). Iodinated proteins were shown to produce thyroidal effects in tadpoles by Morse (1914), Lenhart (1915), and Rogoff and Marine (1916, 1917). Inasmuch as iodinated proteins were not believed at that time to contain a true thyroidal substance, however, these results were generally considered to indicate a lack of specificity for this reaction. More recent investigations (Romeis, 1923; Gaddum, 1927; Lein, 1937; Wokes, 1938; Reineke and Turner, 1942) indicate that the test is relatively specific, little or no response being produced by diiodotyrosine or iodide. It is of interest that thyroid will induce metamorphosis in *Axolotl*, a species that normally does not undergo full metamorphosis even in the adult stage (Uhlenhuth, 1919). The dose-response relationship is too variable, however, for use in quantitative assays (Zavadovsky and Zavadovsky, 1926). Lenhart (1915) and Rogoff (1917) proposed the use of tadpoles for standardizing thyroid preparations. Romeis (1923) proposed that the assay response be based on the minimal dosage of thyroid capable of inducing metamorphosis in a batch of tadpoles after a single feeding. Of the species available, the most extensive studies on the use of tadpoles for thyroid assay have been conducted with *Rana temporaria* (Gaddum, 1927; Wokes, 1938; Deanesly et al., 1945), *Rana pipiens* (Reineke and Turner, 1942) and *Xenopus laevis* (Deanesly and Parkes, 1945). In Calcutta, Dutt and Mukerji (1942) reported that *Rana tigrina* and *Bufo melanosticus* yielded

satisfactory results in thyroxine assays. In this discussion description of detailed assay procedures will be restricted to the first three species.

2. Assays with Rana temporaria

Gaddum (1927) first used *Rana temporaria* tadpoles for the quantitative assay of thyroid substance. Batches of 12 tadpoles were used for each test. After 24–48 hr. exposure to the test substance the tadpoles were transferred to tap water, after which their lengths were measured at intervals until appreciable shortening of the body length occurred. It was reported that the decrease in body length bore a rough quantitative relationship to the dose. Wokes (1938) noted that the percentage decrease in body length of *Rana temporaria* tadpoles exposed to thyroid substance varied directly with the logarithm of the dose. Tadpoles, 20–28 mm. in length were measured and placed in individual test tubes containing 1–2 mg. of thyroid in suspension in 20 ml. of solution. Forty-eight tadpoles were placed on each of 3 dosage levels of test preparation, and a similar series was set up on a standard powder at the same time. After 24 hr. exposure the tadpoles were changed to fresh water, and the water was changed daily thereafter. Final measurement was made when the body length had decreased 15–30%. It was estimated that if 48 tadpoles were used on each of 3 dose levels for both the standard and the test preparations, the error would not exceed 30% at the 5% level of significance. It was noted that the sensitivity of *Rana* tadpoles increases with age, and further, that excessive doses must be avoided in order to keep within the sensitive range for a quantitative response. The use of *Rana temporaria* tadpoles for the assay of thyroactive iodinated proteins was investigated by Deanesly *et al.* (1945). Tadpoles were dosed with iodinated protein placed in suspension in their water for 1 day, kept at a temperature of 25–27°C., and measured at the beginning of the experiment and at the end of the third day. It was concluded that the test can give valuable indications of the thyroidal activity in a preparation, but that it is quantitatively unsatisfactory. An important limitation to use of *Rana temporaria* tadpoles for thyroid assays is the fact that the supply is restricted to the natural spawning season occurring in early summer.

3. Assays with Rana pipiens

As in other species of frogs found in the temperate zone, the natural breeding season of *Rana pipiens* is restricted to a short period in early summer. Although it was generally agreed that ovulation could be induced by frog pituitaries (Rugh, 1934) but not by mammalian gonado-

tropic hormones (Creaser and Gorbman, 1939; Langan, 1941), Wright and Hisaw (1946) reported success with a combination of FSH and LH extracted from sheep pituitary glands. Thus by the artificial culture of tadpoles in the laboratory, the useful season of this species can be extended considerably.

A. BREEDING AND REARING OF *Rana pipiens* TADPOLES

The artificial culture of frog tadpoles was first reported by Rugh (1934) and was used subsequently by Reineke and Turner (1942) to obtain tadpoles for use in thyroid assays. Well-developed ova are normally present in this species when they begin to hibernate in the fall. From September to May such frogs may be stored in the refrigerator at 5° to 10°C. To induce ovulation females are injected intraperitoneally with 2–4 frog pituitaries that have been macerated in distilled water. The female is then partially submerged with water in a small container held at ordinary room temperature. Ova will usually be released 24–72 hr. after injection. When the release of ova is noted, a male frog is sacrificed, his testes are removed, and a sperm suspension is made by macerating the testes in about 15 ml. of tap water. The suspension should show good sperm concentration and motility by microscopic examination. After the sperm suspension has stood at room temperature for about 20 min., the eggs are stripped from the female directly into it. Sufficient water is added barely to cover the eggs, and they are allowed to stand, with gentle agitation occasionally, for about one-half hour. Eggs that become fertilized rotate so that their white poles face downward. After fertilization, the eggs are flooded with water that is aerated continuously through an aerating thimble or sintered gas dispersion disk, and held at 20–22°C. until hatching. At this temperature, the larvae emerge by the third to fifth day. The tadpoles are kept in shallow aquaria containing a good growth of water plants and are fed on a dried liver powder. For this, fresh liver is cut into ½-in. cubes, dropped into boiling water, drained, and run through a meat chopper. It is then mixed with an equal part of wheat flour to form a paste. This is dried and ground to a powder, which will keep indefinitely without spoilage. A small amount of the powder is placed in the aquaria daily. Tadpoles can be used for assays when the rear limb buds begin to form. In *Rana pipiens* this occurs at the 18- to 20-mm. stage.

B. ASSAY TECHNIC

Application of *Rana pipiens* tadpoles to the assay of synthetic thyroproteins has been described in some detail by Reineke and Turner (1942). Tadpoles responded readily when the test powder was placed on the

surface of the water or when a solution of thyroprotein was injected in the body cavity. In view of the results reported by Deanesly *et al.* (1945) and Deanesly and Parkes (1945) in *Rana temporaria* and *Xenopus*, it appears likely that *Rana pipiens* would also respond if exposed to a coarse suspension of thyroprotein in sufficiently high concentrations. Injections are done quite simply, however, and have the merit of insuring that each tadpole receives the prescribed dosage. In assays conducted by this method, large tadpoles collected in the spring can be injected readily with $\frac{1}{10}$ ml. of solution from a 1-ml. tuberculin syringe. For the smaller, laboratory-reared tadpoles, a micro-injection apparatus, capable of delivering accurately 0.01 ml. of solution is required. The sensitivity to thyroidal stimulation increases with age and also with increasing temperature. Consequently it is essential that tadpoles selected for an assay be at a uniform stage of development and that the temperature be uniform for all groups. Among the several possible end points, the decrease in body length appears to be the most satisfactory index of response in this species. On a graded series of dosages, the percentage decrease in body length increases directly as the logarithm of the dose. For this relationship to apply, however, the dosage must be kept within the amount required to produce approximately a 5–30% decrease. With the more potent preparations of synthetic thyroprotein, the effective dosage will usually fall in the range of 5-20 μg. per tadpole. For quantitative results it is necessary, as in other methods, to determine the potency of the test preparation in comparison with a standard. Since the sensitivity of tadpoles varies from time to time, a new reference curve must be run with every set of assays. At least 3 dose levels, set up in geometrically increasing amounts, such as 5, 10, and 20 μg., with 10 to 20 tadpoles per group, are needed for a satisfactory curve. In cases where it is desired to know the relative potencies of a series of preparations, it is often convenient to administer the same amount of each preparation into separate groups of 10 to 20 tadpoles run at the same time, and merely take the average percentage decrease in each as a rough measure of the activity. This technic was found particularly useful by the authors in developing methods for the formation of synthetic thyroproteins.

4. Assays with Xenopus Tadpoles

Xenopus laevis, the South African clawed frog, is unique among amphibian species so far studied in that ovulation and the mating reaction can be induced under laboratory conditions by injection with mammalian gonadotropins. For this reason the species has been used very effectively for pregnancy diagnosis. The extensive work in this field has been

reviewed in the monograph by Weisman and Coates (1944). Because mating can be induced the year round and tadpoles can be reared successfully, *Xenopus* offers the best possibility of a continuous supply of amphibian larvae for use in assays.

A. BREEDING AND REARING OF *Xenopus* TADPOLES

The induction of oviposition and mating in *Xenopus* by the administration of gonadotropic hormone has been described by a number of investigators (Shapiro, 1939; Gasche, 1943; Weisman and Coates, 1944; Deanesly and Parkes, 1945). As recommended by these workers, the male and female are both injected with 250–500 I.U. of chorionic gonadotropin and placed by pairs in small glass aquaria at a temperature of 20°–25°C. The use of a grating on the floor of the aquarium prevents the animals from eating their own eggs. Deposition of the eggs is completed in 20–24 hr., at which time the breeders are removed. At 25°C., hatching is completed in about 2 days. Three to four days later the tadpoles begin to swim about. At this time they are transferred, by means of a dropping pipet, to a larger aquarium or jar. The water temperature is quite critical and should be maintained at 20–25°C. As reported by Deanesly and Parkes (1945) *Xenopus* tadpoles thrive on the liver powder described in an earlier section for *Rana pipiens* tadpoles. The amount fed should be adjusted so that the aquarium will clear up in about 24 hr. They have also shown that aeration or changing of the water, unless it becomes foul, is unnecessary, since tadpoles of this species apparently have a low oxygen requirement. For details of metamorphosis and rearing of young frogs, reference should be made to the reports cited at the beginning of this section. Adult breeding stock is maintained in good condition if kept in an aquarium at room temperature and fed 2 or 3 times a week on small pieces of raw liver. They may be mated at intervals of 4–6 weeks.

B. ASSAY TECHNIC

Detailed studies on the use of *Xenopus* tadpoles have been reported by Deanesly and Parkes (1945a, b). Tadpoles were used for assays 2–4 weeks after hatching, when they had reached a length of 20–25 mm. Iodinated protein or thyroid was placed in suspension in the water; thyroxine was given in slightly alkaline solution. A number of possible end points indicating thyroidal activity were studied, of which the eruption of the front limb buds was considered the most suitable for quantitative work. The test appears quite specific for thyroidal activity, giving no response to KI and only a negligible response to diiodotyrosine when administered in amounts that would be found in iodinated proteins.

However, in comparisons of L- and D,L-thyroxine the activity ratio between the two substances was reported to be 1:1 instead of 2:1, as noted by Reineke and Turner (1945a) in *Rana pipiens* tadpoles. Just as with tadpoles of other species, *Xenopus* were found to vary in sensitivity with the environmental temperature, best results being obtained at 25°-27°C. In the assay method recommended by Deanesly and Parkes, five tadpoles 18-25 mm. in length are placed in 200 ml. of water in a 250-ml. beaker, to which the given dose of test substance has been added as a fine suspension. After 3 days, the tadpoles are changed to fresh water. The response is assessed at the end of the seventh day, as the percentage of tadpoles showing eruption of one or both front legs. Groups of 10 or 20 tadpoles are used at each of 2 or more dose levels. As in the other assays described, it is necessary to use a reference preparation, and the activity of the test specimens is expressed in terms of this standard. In evaluating the activity of a preparation, the percentage response is converted to probits and then plotted against log dosage. The effective dosage concentrations with the usual iodinated protein preparations fall within the range of approximately 0.2-1.0 mg./250 ml. of solution. Before a final assay is carried out, preliminary tests are run to determine dosages that will give one point above and one point below the 50% response line. Deanesly and Parkes state that if the potency of the standard is expressed as 1.0 and the potency of the unknown preparations in terms thereof, the limits of error with varying numbers of tadpoles used per substance, where the slope is not usually less than 5.0, will be approximately as follows:

No. of Tadpoles per substance	Limits of error (P = 0.95)
20	0.66-1.51
30	0.72-1.40
40	0.75-1.34
80	0.81-1.23

It is of considerable interest that tests of the ability of iodinated proteins to induce metamorphosis in tadpoles (Deanesly and Parkes, 1945b) showed good agreement with the ability of the same preparations to stimulate lactation in cows.

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The Chemical Assay of Thyroxine and Other Substances with Thyroidal Activity

BY ROSALIND PITT-RIVERS

CONTENTS

I. INTRODUCTION

1. Historical

The chemical assay of the active principle of the thyroid gland has presented difficulties since it was first attempted, and in spite of much work that has been done, these difficulties have not yet been entirely overcome. In this chapter, both the principles on which such assays are based and the experimental technics described by workers in this field will be given in some detail, so that the relative merits of the different methods may be discussed. The problems of biological assay were described in Chapter XIX.

The historical development of the knowledge of the thyroid cannot be described fully here; only those discoveries which have a bearing on the problems of chemical assay will be mentioned. The first of these discoveries was the association between the thyroid gland and iodine and was suggested by the work of Fyfe in Edinburgh (1819) and Coindet in Switzerland (1820), six years after the discovery of iodine. Fyfe demonstrated the presence of iodine in burnt sponge, an ancient remedy for goiter; Coindet showed that goiter might be treated successfully by giving iodine to the goitrous patient.

Some thirty years later, Chatin (1850) began a series of experiments which were designed to show the relationship between iodine deficiency and the incidence of goiter; the work entailed the determination of iodine in air, water, soil, foodstuffs, and vegetation over a wide area of France, and his findings led Chatin himself to the conclusion that iodine deficiency and goiter are related; but the analytical methods for determining iodine then available were so crude and led to such ambiguous findings, that Chatin's theory became for a time discredited.

2. *Discovery of Iodine in the Thyroid*

In 1896, Baumann discovered iodine in the thyroid; this iodine could not be separated from the gland substance by the use of solvents and was therefore thought by Baumann to be combined with the thyroid protein. He later showed (Baumann and Roos, 1896) that this was indeed so: the iodine could only be removed from the gland together with a protein which was soluble in dilute salt solutions and was precipitated with this protein by the action of dilute acid and heat.

Meanwhile, the elucidation of the function of the thyroid gland, which had progressed over the fifteen years preceding Baumann's discovery, culminated in the finding by Murray (1891) that the condition of myxedema could be dramatically relieved by injection of thyroid extract. In the following year it was shown that thyroid substance could be administered advantageously and more easily by mouth; thereafter the therapeutic use of thyroid preparations became widespread, and by 1898 a short description of the preparation of desiccated thyroid powder had appeared in the British Pharmacopoeia.

The Pharmacopoeia of the United States VIII (1905) contains a description of the preparation of desiccated thyroid powder, together with a qualitative test for organically bound iodine. Here for the first time, the presence of iodine is officially given as an essential requirement for the potency of any thyroid preparation. The test described entails the alkaline incineration of the thyroid gland in the presence of nitrate; the residual ash is dissolved in water, acidified, and treated with nitrite; the iodine thus liberated is shaken out into chloroform to which it imparts a violet color. The test should be negative when it is applied to the non-incinerated gland.

In 1910, Hunter reviewed the current methods of iodine analysis, and exposed many errors both of principle and of technic; at the same time Hunter described a new method for the determination of iodine which is still used in principle at the present time. The method was as follows. The organically bound iodine in the substance to be assayed was liberated (as a mixture of iodide and iodate) by total alkaline incineration with a mixture of sodium and potassium carbonates and potassium nitrate; the iodide was oxidized by chlorine in dilute acid solution to iodate, and in the presence of excess iodide and acid the iodate was converted to free iodine, according to the equation:

$$HIO_3 + 5KI + 5HCl = 3I_2 + 3H_2O + 5KCl.$$
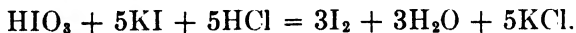
The iodine was then determined by titration with standard sodium thiosulfate using starch as indicator.

In 1914, Kendall published a modification of Hunter's method for iodine determination in the thyroid, in which certain improvements were described. Sodium hydroxide was used for the incineration instead of the carbonate mixture; small amounts only of potassium nitrate were used for the final oxidation of organic matter, and bromine was substituted for chlorine for the final oxidation of iodide to iodate.

3. Isolation of the Active Principle of the Thyroid

The first important advance was made by Kendall (1913); he showed that if thyroid powder was treated with sodium hydroxide at a raised temperature a fraction could be obtained which contained over 50% of the total iodine of the thyroid and was active in the treatment of myxedema. Later Kendall (1915) used alcoholic sodium hydroxide as a hydrolytic agent and obtained an active fraction from thyroid powder which was separated from the inactive fraction by acid precipitation. This fraction contained ten times as much iodine as the starting material. In 1919, Kendall achieved the isolation of a crystalline acid-insoluble compound containing 65% of iodine by the hydrolysis of fresh thyroid glands with 5% sodium hydroxide. Purification of the compound was extremely laborious and involved great losses, the yield from three tons of pigs thyroids being only 33 g. Kendall's acid had a physiological activity similar to that of the whole thyroid; he named it thyroxine.

4. The Constitution of Thyroxine

In 1926, Harington began his experiments on the thyroid hormone which were to lead to the elucidation of its structure. The first paper (Harington, 1926a) described a greatly improved method of isolating thyroxine from the thyroid by substituting a two-stage baryta hydrolysis for Kendall's sodium hydroxide hydrolysis. The total yield of thyroxine isolated was 0.125% of the desiccated gland. In the same year, Harington (1926b) showed by degradation and by synthesis that thyroxine from which the iodine atoms had been removed by reduction was β-[4-(4'-hydroxyphenoxy)phenyl]-α-aminopropionic acid; Harington named this amino acid thyronine. In the following year Harington determined the orientation of the iodine atoms in thyroxine (Harington and Barger, 1927) and showed that thyroxine is β-[3,5-diiodo-4(3',5'-diiodo-4'-hydroxyphenoxy)phenyl]-α-aminopropionic acid:
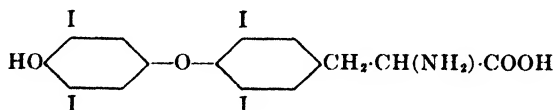
Three years later, Harington and Randall (1929a) isolated 3,5-diiodo-tyrosine from the thyroid, the isolation depending upon the separation of acid-insoluble (thyroxine) and acid-soluble (diiodotyrosine) iodine-containing compounds after preliminary hydrolysis; according to the authors, these two amino acids accounted for all the organically bound iodine in the thyroid.

Now the biological inactivity of diiodotyrosine had already been amply demonstrated; Strouse and Voegtlin (1909) had found that diiodotyrosine was without effect on the nitrogen metabolism and blood pressure of dogs, and had no curative action on cretinism and myxedema; Gaddum (1929, 1930) had found that it had no effect on oxygen consumption in rats. Diiodotyrosine did not accelerate tadpole metamorphosis (Zawadowsky, Titajev, Perelmutter, and Raspopowa, 1927; Gaddum, 1927–28) unless administration of large doses was prolonged (Romeis, 1923; Abderhalden, 1924). The separation of thyroxine and diiodotyrosine (Harington and Randall, 1929a) mentioned above, together with the biological inactivity of diiodotyrosine forms the basis for the first method of chemical assay of the thyroid hormone itself (Harington and Randall, 1929b). The method, which will be described in the experimental section, was adopted in principle for the standardization of commercial thyroid preparations by the British Pharmacopoeia 1932, Addendum 1936, and is still recommended by the British Pharmacopoeia today.

5. Separation of Thyroxine by Butanol Fractionation

In 1932, Leland and Foster discovered that thyroxine could be separated from diiodotyrosine in alkaline solution by extraction with *n*-butanol. Approximately 90% of the thyroxine passed into the butanol layer, taking with it only a small fraction of the diiodotyrosine, and this could be removed from the butanol with an alkaline washing. This method of separation was applied to sodium hydroxide hydrolysates of thyroid preparations, and the thyroxine content was determined by iodine estimations on the butanol extracts. Later Blau (1933) showed that a better separation of thyroxine and diiodotyrosine could be effected by extracting the hydrolysate of thyroid gland with *n*-butanol at *p*H 3.5 to 4. Such an extraction removed the whole of the thyroxine from the aqueous phase, as well as a large proportion of the diiodotyrosine, but the latter could be removed by washing the butanol extract with $4N$ sodium hydroxide containing 5% of sodium carbonate.

It is upon these two properties of thyroxine, namely its acid insolubility and its solubility in *n*-butanol that the separation of thyroxine from diiodotyrosine is based in all the methods described in the experi-

mental section.   It will be seen that the butanol fractionation method
is now the one most widely used.

6. Artificial Thyroproteins Possessing Biological Activity

Meanwhile, a new problem has arisen in the last ten years as a result
of Ludwig and von Mutzenbecher's discovery (1939) that the iodination
of certain tyrosine-containing proteins in a slightly alkaline medium
gave products possessing thyroidal activity from which thyroxine
could be isolated after alkaline hydrolysis.   Ludwig and von Mutzen-
becher studied certain factors governing the optimum conditions for
thyroxine formation and found that the relationship between the iodine
and the thyroxine in the protein was of critical importance.   Small
amounts of added iodine led to the formation of only small amounts of
thyroxine, an optimum being reached when about 4 atoms of iodine per
molecule of tyrosine had been added.   Greater amounts of iodine than
this led to the formation of less active or inactive compounds, which on
hydrolysis gave only small yields of thyroxine together with tarry
products with no biological activity.

Many other workers in this field have studied the conditions affecting
thyroxine formation, notably Reineke and Turner (1942, 1945, 1946) in
America; Roche, Michel, and Lafon (1947); Roche and Michel (1947) in
France, and a group of workers in England organized by the Agricultural
Research Council to study the effect of artificially iodinated proteins on
milk production in cows.   Reineke and Turner in particular have
investigated the conditions required for the preparation of highly active
iodinated proteins; they have studied the effect of temperature, metal
catalysis and the variation of added iodine on the biological potency of
their preparations as measured by the tadpole metamorphosis test and
by their effect on the metabolism of guinea pigs; the effect of active
preparations on lactation has also been studied.

In the experiments organized by the British Agricultural Research
Council, iodinated proteins were prepared (according to Ludwig and
von Mutzenbecher) and assayed chemically for total iodine and acid-
insoluble (thyroxine) iodine by Pitt-Rivers and Randall (1945) before
experiments on the effect of these preparations on lactating cows were
undertaken (Blaxter, 1945).   Many discrepancies between the chemical
assay and the biological activity become apparent, since products with
a high acid-insoluble iodine content were often almost devoid of activity.
This showed that during iodination, acid-insoluble iodinated products
other than thyroxine must have been formed.   This was amply con-
firmed by Deanesly and Parkes (1945) who found that they were unable
to correlate the acid-insoluble iodine content of many iodinated proteins

with their biological activity as measured by the tadpole metamorphosis test. The identity of acid-insoluble iodine and thyroxine iodine no longer existed, and a new chemical method was needed which would be specific for thyroxine estimation.

It cannot be too strongly emphasized that such a specificity is an essential requirement of any chemical assay when it is applied to artificial thyroproteins. How far the newer methods conform with this requirement will be discussed after the methods have been described.

7. Problems of Hydrolysis

In the chemical assay of thyroxine, the method and degree of hydrolysis of the protein under investigation are of fundamental importance; mild hydrolysis such as that recommended by Harington and Randall (1929b) may lead, according to other workers, to high thyroxine values being obtained as a result of iodine assay, and Leland and Foster (1932) advocate a much longer hydrolysis with stronger alkali; on the other hand, the action of alkali or acid on thyroxine always leads to some destruction, and losses will occur under the best conditions. The problem is resolved into a search for a hydrolytic agent powerful enough to free thyroxine completely from peptide linkage which at the same time will effect the minimum amount of destruction. Acid hydrolysis of the thyroid gland was early shown to cause extensive destruction of thyroxine (this is true even in the case of pure derivatives of thyroxine). The stability of thyroxine toward different alkalis though much greater than toward acids, is variable. Harington (1926a) showed that better yields of thyroxine could be isolated from thyroid by substituting baryta for sodium hydroxide as the hydrolytic agent, and some workers (Blau, 1935; Roche and Michel, 1947–48) have advocated the use of relatively dilute baryta solution for the hydrolysis of thyroid and iodinated proteins. Attempts have been made to determine the amount of destruction of thyroxine during hydrolysis by recovery experiments on thyroxine added to proteins before hydrolysis (cf. Leland and Foster, 1932; Taurog and Chaikoff, 1946a), but the interpretation of the results is open to the objection that the destruction of free thyroxine is not necessarily the same as that of thyroxine which is being liberated from protein combination; indeed it is generally thought that free thyroxine is the more unstable and any calculations of losses occurring during hydrolysis based on recovery experiments will give a maximum value. This uncertainty will give rise to unknown errors in the determination of thyroxine in protein hydrolysates, and an ideal method of chemical assay of thyroxine could be one which involved no hydrolysis, i.e., on the whole protein.

Attempts have been made to use a physico-chemical property of

thyroxine toward this end: Ginsel (1939) measured the absorption spectra of thyroglobulin, diiodotyrosine, and thyroxine, and from his data, estimated that these amino acids were present in thyroglobulin in the proportion of two molecules of diiodotyrosine to one molecule of thyroxine; more recently Reineke and Turner (1942) have attempted to follow the formation of thyroxine during the iodination of proteins from measurements of the absorption spectra of the proteins.

Another physico-chemical method for thyroxine assay has been described; Simpson and Traill (1946), Simpson, Johnston, and Traill (1947) and Borrows, Hems, and Page (1949) have described polarographic methods for the determination of thyroxine; this can be done in the presence of diiodotyrosine since the two amino acids give different current-voltage curves. Colorimetric methods have also been described for thyroxine assay; one (Winikoff and Trikojus, 1948) is specific for thyroxine and other tetraiodohydroxydiphenyl ethers (diiodophenolic derivatives do not give the color), but most are non-specific (Morton and Chaikoff, 1943; Roche and Michel, 1946–47, etc.) and depend upon the separation of thyroxine from diiodotyrosine.

The methods of which the different principles have been outlined above will now be described in some detail.

II. Methods of Chemical Assay of Compounds Having Thyroidal Activity

1. Assay of the Thyroid Gland by Total Iodine Determination. Kendall (1914)

Thyroid powder (0.5 g.) is evaporated down in a nickel crucible with 5–6 ml. of 30% sodium hydroxide together with a little added iodine-free organic material (gallic acid) to prevent splashing. When the water has boiled off, heating is continued to incinerate all the organic material. At first considerable foaming occurs, but this subsides as incineration proceeds and eventually bubbling will cease. Five to ten milligrams of KNO_3 is added (if necessary this is repeated) to complete oxidation of organic material, and the melt is allowed to cool. It is then dissolved in water (200 ml. in all) and transferred quantitatively to a flask; 10% sodium bisulfite (1 ml.) is added, and a few drops of methyl orange, and the solution is just acidified with phosphoric acid (until the indicator is a faint pink). A few drops of bromine are added and the solution is boiled for 8–10 min. to expel the bromine; any residual traces are removed by adding a few drops of 5% sodium salicylate solution. When the solution is cool 10% KI solution (5 ml.) is added, and if necessary a little phos-

phoric acid.   The iodine liberated is titrated with $N/200$ sodium thiosulfate solution, using starch as the indicator.

2. Assay of Thyroxine Iodine

A. SEPARATION OF THYROXINE BY ACID PRECIPITATION

i. *Harington and Randall (1929b)*.   Desiccated thyroid gland is boiled under reflux for 4 hr. with 10 parts of N NaOH; the hydrolysate while still hot is filtered from inorganic material and an aliquot of the filtrate is analyzed for total iodine.   The remainder is acidified with 50% sulfuric acid to pH 5 and is allowed to stand overnight for complete separation of the insoluble fraction.   The suspension is filtered and the acid-soluble iodine is determined on an aliquot of the filtrate.   The acid-insoluble or thyroxine iodine is calculated by the difference between the two determinations.   Iodine determinations are done by Kendall's (1914) method as described by Harington and Randall (1929b) and by Harington (1933, p. 192).   The alterations from Kendall's method are slight (5 g. NaOH is used + 5 ml. water for the incineration of 0.5 g. thyroid gland), but the descriptions given in both of Harington's references are compressed into half a page and therefore are easy to follow.

ii. *Doery (1945)*.   This author has examined the assay of thyroid preparations when the hydrolyses are carried out according to Harington and Randall (1929b) and according to Leland and Foster (1932) (prolonged boiling of thyroid with $2N$ NaOH).   Doery concludes that the method of hydrolysis of Harington and Randall is inadequate and does not lead to reproducible results, and that even if the more drastic hydrolysis of Leland and Foster is employed, unreliable thyroxine iodine values are obtained by the acid precipitation method of assay, since iodide may be adsorbed on the precipitate and will therefore appear as acid-insoluble. Other factors which may affect the determination of thyroxine are also considered by Doery, i.e., the deleterious effect of lactose (used as excipient in desiccated thyroid powder or tablets) on thyroxine during hydrolysis and the interference caused by silica (from prolonged alkaline hydrolysis in glass flasks) in the iodine determinations.   Nickel flasks are recommended to overcome this difficulty.

iii. *Rivière, Gautron, and Thély (1947)*.   Total iodine is determined by alkaline fusion of the thyroid gland or iodinated protein to be assayed. A different sample, 0.25–0.5 g. is hydrolyzed by boiling under reflux for 6 hr. with 10% baryta; the hydrolysate is acidified to pH 4.5 exactly, and allowed to stand overnight in the ice-box.   The precipitate is separated by filtration and washed with water which has been brought to pH 4.5

with HCl; the whole precipitate is determined for thyroxine iodine, and an aliquot of the filtrate is determined for non-thyroxine iodine. Iodine is determined by the method of Fabre and Penau (1933). These authors criticize Harington and Randall's (1929b) method because they state that iodine will be lost on the precipitate of inorganic material when the alkaline hydrolysate is filtered before total iodine assay; variable losses of iodine will occur owing to adsorption.

B. SEPARATION OF THYROXINE BY BUTANOL FRACTIONATION

i. *Leland and Foster (1932).* Desiccated thyroid gland (1.25 g.) is boiled under reflux for 18 hr. with $2N$ NaOH (100 ml.). The cooled hydrolysate is extracted twice with an equal volume of n-butanol and the combined butanol extracts are washed with an equal volume of N NaOH. This washing is re-extracted with half its volume of butanol. After evaporation of the butanol extract to a low volume, the residue is transferred quantitatively to a nickel crucible containing a few milliliters of 50% NaOH and assayed for iodine by a modification of Kendall's method. The authors found that the mean thyroxine iodine content of 52 human thyroids assayed in this way was 25% of the total iodine content. Leland and Foster criticized the method of hydrolysis used by Harington and Randall (1929b) on the ground that it is inadequate and gives high values of thyroxine iodine; in support of this criticism, they showed that if an acid-insoluble precipitate from a thyroid hydrolysate performed according to Harington and Randall were further hydrolyzed for 18 hr. with $2N$ NaOH and fractionated into n-butanol, the original value for acid-insoluble iodine was nearly halved. Leland and Foster also did recovery experiments of added thyroxine to casein which was hydrolyzed as described above. Recoveries (as thyroxine iodine) were of the order of 80%.

ii. *Blau (1933, 1935).* Dried defatted thyroid is hydrolyzed (Blau, 1933) by the method of Leland and Foster (1932); the cooled hydrolysate is brought to pH 3.1–3.5 with sulfuric acid and extracted once with an equal volume of n-butanol. The butanol extract is washed with an equal volume and then half its volume of $4N$ NaOH containing 5% sodium carbonate. After evaporation of the butanol layer, the iodine content of the residue is determined by the method of Kendall (1914).

(Blau, 1935). Desiccated thyroid gland (1 g.) is hydrolyzed with 8% baryta (50 ml.) for 6 hr. The cooled hydrolysate is brought to pH 3.5–4 with HCl, extracted with butanol, and assayed as in the 1933 method. Blau compared the values for thyroxine iodine obtained by baryta hydrolysis with those obtained by Leland and Foster and found that they were consistently higher. The thyroxine iodine was about

30% of the total iodine. Blau also determined the thyroxine iodine content of thyroid preparations by the acid precipitation method of Harington and Randall after baryta hydrolysis of the glands; he found that the acid precipitation method gave values in fairly good agreement with those obtained by the acid-butanol extraction method.

iii. *Reineke, Turner, Kohler, Hoover, and Beezley (1945).* Iodinated protein (1 g.) is heated at 100°C. for 18 hr. with hydrated baryta (3.2 g.) and water (6.4 ml.). The cooled hydrolysate is diluted with water (25 ml.) and the precipitated barium salts are allowed to settle. The supernatant liquid is decanted into a 100 ml. volumetric flask, and the barium salts are decomposed with 3.5N HCl (5 ml.) in the presence of *n*-butanol (2 ml.). This product is transferred to the volumetric flask, and the contents are diluted to the mark. A 10–20 ml. aliquot of this suspension is adjusted to *p*H 3.5 and extracted with *n*-butanol (equal volume). The butanol extract is washed twice (equal volume and then half volume) of 4N NaOH containing 5% sodium carbonate, evaporated on a steam bath at atmospheric pressure, and the iodine content of the residue is determined according to Harington (1933, p. 192). The authors were able to recover added thyroxine quantitatively from known solutions by this extraction procedure; when thyroxine was heated for 20 hr. with 40% baryta recovery was still 93%. The hydrolysis of iodinated proteins by Blau's method (boiling for 6 hr. with 8% baryta) led to high thyroxine values if these were compared with the biological activity of the proteins as measured by metabolic stimulation in guinea pigs.

iv. *Taurog and Chaikoff (1946a).* Single rats thyroid are hydrolyzed by heating at 100°C. for 15 hr. with 2N NaOH (1 ml.). The hydrolysates are brought to *p*H 3.5–4 with 6N H_2SO_4 and extracted twice with *n*-butanol. The combined butanol extracts are washed twice with 4N NaOH containing 5% sodium carbonate and evaporated under diminished pressure in a stream of CO_2. The iodine content of the residue is determined by a modification (Taurog and Chaikoff, 1946b) of the Chaney (1940) method, which will be described in Section III. Non-thyroxine iodine is determined on the aqueous phase (after butanol extraction) combined with the alkaline washings. Taurog and Chaikoff confirmed Blau's findings (1935) that hydrolysis of thyroid gland with 8% hydrated baryta leads to higher thyroxine values than hydrolysis with 2N NaOH. Nevertheless, they found that baryta hydrolysis led to variable thyroxine values and therefore preferred 2N NaOH as the hydrolytic agent. Recovery experiments on added thyroxine showed that during hydrolysis with 2N NaOH approximately 25% of the thyroxine was destroyed. The destruction of thyroxine liberated from desiccated thyroid gland was

thought to be not so great (15%) and did not increase when hydrolysis was prolonged from 7 to 20 hr. The thyroxine iodine content in single rat thyroids varied from 1–2 μg. and was found to be 23–30% of the total thyroid iodine.

3. Colorimetric Determination of Thyroxine

A. BRAND AND KASSELL (1939)

The method is based on Lugg's (1938) observation that diiodotyrosine is quantitatively reduced to tyrosine by the action of alkaline stannite. Brand and Kassell find in experiments with the pure compounds that 1 mg. of thyroxine yields 0.286 mg. "extra tyrosine," presumably thyronine, and 1 mg. of diiodotyrosine yields 0.716 mg. extra tyrosine after alkaline stannite reduction; a method for the determination of thyroxine and diiodotyrosine in thyroid preparations has been worked out. Thyroid samples (50–100 mg.) are hydrolyzed by heating at 100°C. for 16 hr. with 5N NaOH (2 ml.). The tyrosine and tryptophane contents are determined on the hydrolysates by Lugg's (1937–38) modification of Folin and Ciocalteu's method (1927) (Millon reaction). Other samples of the thyroid preparations (50–100 mg.) are then hydrolyzed with 5.5 N NaOH containing 5% stannous chloride (2 ml.) and the hydrolysates are again determined for tryptophane and tyrosine. After the alkaline stannite hydrolysis, "extra" tyrosine is found. A total iodine determination is made on the thyroid preparation (thyroxine plus diiodotyrosine iodine). From these data (iodine content and extra tyrosine) the thyroxine and diiodotyrosine contents of the thyroid preparations can be calculated from equations derived by the authors. It was found that only small amounts of thyroxine can safely be determined in this way; quantities greater than 2 mg. in their samples are partially precipitated as the insoluble mercury salts and determined as tryptophane. The thyroxine content of thyroid preparations determined by this method was found to be somewhat higher than those obtained by Leland and Foster's (1932) method, but lower than the values obtained by Harington and Randall's (1929b) method.

B. MORTON AND CHAIKOFF (1943)

This method is based on the Kendall-Osterberg (1919) reaction. Many *ortho*-diiodophenols give a pink to purple color when they are treated with nitrous acid in alcohol-HCl solution and then made alkaline with ammonia. Thyroxine and diiodotyrosine give similar colors with this reagent, and this color reaction has been used by Morton and Chaikoff for the determination of small amounts of added thyroxine and

diiodotyrosine in experiments on the isolation of thyroxine and diodo-
tyrosine containing radioactive iodine. The carrier thyroxine and
diiodotyrosine are recrystallized several times and the amount of pure
amino acid present after each crystallization determined; 2–3 mg.
thyroxine is dissolved in alcohol (5 ml.) and treated with $6N$ HCl (0.2 ml.)
and 1% sodium nitrite solution (0.5 ml.). The solution is just brought
to the boiling point and is then cooled and made alkaline by the addition
of concentrated ammonia (0.5 ml.). Aliquots are taken for colorimetric
determination of thyroxine made by comparing the colorimetric readings
obtained with pure thyroxine. Diiodotyrosine may be determined in the
aqueous phase after butanol extraction in a similar manner. Impurities
such as sulfate and acetate were found to interfere with the color.

C. ROCHE AND MICHEL (1946, 1947); ROCHE, MICHEL, AND LAFON (1947b)

These authors have used the Kendall-Osterberg reaction for the
determination of thyroxine and diiodotyrosine in thyroid substance and
in iodinated proteins. Thyroid powder (50–200 mg.) is hydrolyzed by
boiling under reflux with 8% baryta (10 ml.) for 6 hr. After adjusting
the pH of the hydrolysate to 3.5–4, it is diluted to 15 ml. with water and
extracted once with an equal volume of n-butanol. The butanol extract
is washed twice with $5N$ NaOH and evaporated to dryness; the residue
is dissolved in 15 ml. of an HCl-ethanol mixture containing NaCl. The
solution (7.5 ml.) is treated with 1% sodium nitrite solution (1 ml.), and
the mixture is allowed to react for 10 min. at 15°C.; 20% ammonia
(15 ml.) is then added and the color formed is measured in a Pulfrich
photometer within 15 min., using filter S50. The amount of thyroxine
present is determined by comparison with a standard curve obtained
with pure thyroxine.

A method for estimating diiodotyrosine has also been worked out,
using the same color reaction. Monoiodotyrosine does not give a color
with the Kendall-Osterberg reagent, but may be determined colorimetri-
cally with the aid of Millon's reagent. Monoiodotyrosine and tyrosine
give colors with Millon's reagent which are sufficiently different for the
amino acids to be determined in the presence of each other, making use
of selective color filters. Roche, Michel, and Lafon (1947b) have used
the method described above for the determination of thyroxine, diiodo-
tyrosine and monoiodotyrosine in iodinated proteins.

D. MOSER (1947)

This method is based on Komant's (1930; see also 1947) finding that
if thyroxine is coupled with diazotized sulfanilic acid in alkaline solution,
a purple color is produced. Moser couples thyroxine with diazobenzene-

sulfonic acid in sodium carbonate solution; the red color obtained has maximum absorption at 5100 Å. The determination is made on thyroxine solutions containing not more than 10 mg. thyroxine per 100 ml.; 10% sodium carbonate solution (5 ml.) is added to the thyroxine solution (5 ml.) and cooled to 0°C.; freshly prepared cooled diazobenzene sulfonic acid solution (prepared according to Weiss and Ssobolew, 1914) is added and well mixed for 15 sec.—4 min. after the first appearance of the color $3N$ NaOH (2 ml.) is added, and the color is measured in a Pulfrich photometer within 3 min. of the addition of the alkali. Filter L2 is used (between S50 and S53).

E. WINIKOFF AND TRIKOJUS (1948)

This method also depends upon the color produced when thyroxine is coupled with a diazotized amine in alkaline solution. The authors first investigated the color obtained with various diazotized amines and found that N^1-diethylsulfanilamide gave the most satisfactory results. The method finally chosen is as follows: the thyroxine solution containing 0.02–0.1 mg. in $0.1N$ NaOH (2 ml.) is made strongly alkaline by the addition of $2N$ NaOH (1 ml.) and shaken into n-butanol (2 ml.) in a centrifuge tube; $2N$ NaOH (1 ml.) is added to the freshly prepared diazo solution (containing about 4 mg. diazonium chloride), and this is added to the reaction mixture in the centrifuge tube and shaken vigorously for 15 sec.; gentle mechanical agitation is continued for 24 hr. after which the mixture is centrifuged and the butanol layer separated with a Pasteur pipet; to an aliquot of the butanol solution (0.75 ml.) is added N NaOH in ethanol (0.25 ml.), and the color is measured in a Hilger Spekker absorptiometer using the Ilford spectrum green filter. The behavior of a number of compounds was investigated with this reagent in order to determine their possible interference with the determination of thyroxine; diiodotyrosine, 3,5-diiodo-4-hydroxyphenyl lactic acid, diiodothyronine and glutamyl thyroxine gave colors from yellow to pale pink; thyroxamine and thyroxyl diiodotyrosine gave purple colors similar to that given by thyroxine; elsewhere (Saul and Trikojus, 1948) it was found that the lactic acid analog of thyroxine also gave an intense purple color with this reagent.

4. Polarographic Determination of Thyroxine

A. SIMPSON AND TRAILL (1946); SIMPSON, JOHNSTON, AND TRAIL (1947)

The behavior of thyroxine and of diiodotyrosine at the dropping mercury cathode was first investigated by Simpson and Traill in order to determine whether polarographic analysis could be used in the estimation

of these compounds. It was found that under suitable conditions, both compounds were electroreducible in alkaline solution, giving distinct polarographic waves.

Methods. Thyroxine (20 mg.) is dissolved in $0.3N$ Na_2CO_3 in 40% ethanol (20 ml.), and to the solution is added tetramethylammonium iodide (0.2 g.). Oxygen is removed from the solution by bubbling nitrogen through it, and the polarogram is recorded, using a Cambridge polarograph. Current e.m.f. curves show three waves having half-wave potentials of -1.20 v., -1.40 v. and -1.70 v. Under similar conditions, diiodotyrosine in aqueous carbonate solution gives two polarographic waves having half-wave potentials of -1.5 v. and -1.7 v. In aqueous-ethanol carbonate solution diiodotyrosine does not begin to discharge until about -1.3 v. The authors therefore suggest that thyroxine may be estimated in the presence of diiodotyrosine by making use of the first thyroxine wave at -1.2 v.

Simpson, Johnston, and Traill (1947) have studied polarographically the thyroxine content of various iodinated proteins after baryta hydrolysis. The proteins were refluxed for 20 hr. with 10 parts 50% baryta. Active acid-insoluble fractions (not assayed biologically) were obtained which were assayed for thyroxine both chemically (iodine content) and polarographically. The results obtained by the two methods were in good agreement. The authors point out that in the polarographic determination of thyroxine, it is assumed that thyroxine is the only substance in the acid-insoluble fraction of the hydrolysates which will give the known polarogram of thyroxine.

B. BORROWS, HEMS, AND PAGE (1949)

These authors have examined critically a number of chemical methods for thyroxine assay given in the literature and consider that the polarographic method offers much promise. Using a Cambridge polarograph, the conditions for obtaining the most satisfactory polarograms of thyroxine and diiodotyrosine have been determined and the following method is given. Thyroxine is dissolved in 20% isopropyl alcohol containing 1% of tetramethylammonium bromide and $0.5N$ Na_2CO_3. Oxygen is removed from the solution by bubbling nitrogen through it. Thyroxine gives a series of three polarographic steps, the first having a half-wave potential of -1.12 v. Under the same conditions the first diiodotyrosine step has a half-wave potential of -1.51 v. Thyroxine may therefore be determined in the presence of diiodotyrosine since the first step is not masked by the first diiodotyrosine step. Borrows, Hems, and Page found that thyroxine could be determined in the presence of tenfold excess of diiodotyrosine; greater amounts interfered with the thyroxine

determination and must be removed. Purification of the thyroxine by *n*-butanol fractionation according to Leland and Foster (1932) was carried out, and the butanol extracts were examined polarographically. This revealed the presence of considerable amounts of diiodotyrosine, much more than was found by Leland and Foster. The authors also examined polarographically a number of compounds to see whether these would interfere with the determination of thyroxine. They included various phenols, diiodophenols, derivatives of diiodotyrosine, and certain iodinated diphenyl ethers.

Iodinated casein hydrolysates were assayed polarographically for thyroxine after the best conditions of hydrolysis had been determined by experiments; 40% baryta hydrolysis followed by acid-butanol extraction was chosen. The butanol extracts of a number of casein hydrolysates were assayed both chemically (iodine content) and polarographically. If the thyroxine content were calculated from the height of the total thyroxine step (at -1.70 v.) the result was in good agreement with the result obtained by iodine analysis. The thyroxine content calculated from the height of the first thyroxine step (at -1.12 v.) was only one-third to one-half of that obtained chemically. This value is regarded by the authors as the "true" thyroxine value. The total thyroxine obtained chemically could include some diiodotyrosine extracted with the butanol. The method was tested by recovery experiments on thyroxine added to casein hydrolysates and iodinated casein hydrolysates. Recovery ranged between 75 and 100%. As regards the specificity of the method, the authors point out that any tetraiodohydroxydiphenyl ether (with the same orientation of iodine atoms as thyroxine) will be included in the polarographic determination of thyroxine.

5. Attempts to Determine Thyroxine in Proteins from Absorption Spectra Data

A. GINSEL (1939)

Determinations of the ultraviolet absorption spectra of thyroxine, thyronine, tyrosine, diiodotyrosine, and thyroglobulin at an alkaline *p*H led Heidt (1936) to the conclusion that the differences were too small to allow for the identification of the compounds in the presence of each other. Ginsel redetermined the absorption spectra of diiodotyrosine thyroxine and thyroglobulin at acid and alkaline reactions and described a method of estimating quantitatively the diiodotyrosine and thyroxine present in thyroglobulin, making use of their differential extinction coefficients (this constant is defined as the difference between the alkaline

and acid molecular extinction coefficients). By comparing thyroglobulin (experimental) curves with theoretical curves obtained from known mixtures of diiodotyrosine and thyroxine Ginsel concluded that these are present in thyroglobulin in the ratio of two molecules of diiodotyrosine to one molecule of thyroxine.

B. REINEKE AND TURNER (1942)

These authors have studied the absorption spectra of iodinated casein preparations containing progressively increasing amounts of iodine and have compared the curves obtained with absorption spectra data for pure thyroxine; it has been shown that increasing iodination caused a shift in the maxima and minima of the curves toward the longer wavelengths (cf. Marenzi and Villalonga, 1941), indicating the presence of increasing amounts of thyroxine in the iodinated proteins. However, the absorption spectra curves of excessively iodinated proteins, possessing lower biological activity, most nearly approached that of pure thyroxine; the authors have provided a possible explanation for this phenomenon.

6. Suggested Chemical Assay of Iodinated Proteins by the Isolation of Thyroxine

A. PITT-RIVERS (1949)

Pitt-Rivers (1948) found, when examining the alkaline hydrolysates of iodinated proteins of low biological activity and high acid-insoluble iodine content that very little thyroxine could be isolated from them by the Leland and Foster technic (1932). Further experiments on the relationship between thyroxine content and biological activity were indicated. For this purpose, six iodinated proteins with varying acid-insoluble iodine contents and biological activities (as measured by the tadpole metamorphosis test by Deanesly and Parkes, 1945) were chosen. These were iodinated casein and iodinated "Ardein" preparations. The following simple method of hydrolysis and isolation was used. The iodinated protein (30 g.) was boiled under reflux in an electric heating mantle with hydrated baryta (100 g.) and water (200 ml.). The precipitated barium salts were collected by filtration of the hot hydrolysate and decomposed with 1% NaOH solution and sodium sulfate by the method of Harington (1926a). The acid-insoluble precipitate from this fraction was combined with a small acid-insoluble fraction obtained by acidifying the filtrate with HCl. After drying *in vacuo*, the crude material was extracted twice with ether, dissolved in the minimum amount of boiling $N/10$ Na_2CO_3, filtered if necessary, and cooled. The sodium salt of thyroxine separated slowly and crystallization was pro-

longed for 72 hr. at 0°C. The thyroxine sodium salt was collected at the centrifuge and crystallized by dissolving in the same volume of boiling $N/10$ Na_2CO_3 as before and acidifying with acetic acid (cf. Taurog and Chaikoff, 1948). The thyroxine contents of the proteins were found to be proportional to their biological activity.

III. The Determination of Iodine in Organic Combination

The determination of organically bound iodine is a two stage process involving the liberation of iodine in an inorganic form by destruction of the organic material followed by the determination of the inorganic iodine. A large number of methods have been described within the last few years. These depend in the main upon two principles: (1) the conversion of the inorganic iodine liberated by combustion to iodate, which is determined by the reaction first described by Hunter; (2) conversion of inorganic iodine to iodide which is determined by means of the reaction first described by Sandell and Kolthoff (1937), i.e., the catalytic action of iodide ions on the reduction of ceric ions by arsenious acid. Numerous modifications based on these principles have been published both for the micro and semimicro determination of iodine and different methods for the destruction of organic material have been used; alkaline incineration may be achieved with sodium or potassium hydroxide, carbonates, and mixtures of carbonates and nitrates. Acid oxidation may be performed with sulfuric and chromic acids, sulfuric acid and permanganate, etc. After acid digestion the iodine is reduced to HI with oxalic or phosphorous acids and distilled. The methods are far too numerous to be described in detail—reference will merely be made to the principal modifications.

1. Semimicro Methods

The principle and details of Kendall's (1914) method have already been described. This method, Harington and Randall's modification (see also Harington, 1933), and other modifications have been used by many workers including von Fellenberg (1923, 1930); Leland and Foster (1932); Cuny and Robert (1933); Blau (1933;) Doery (1945) (see also Doery, 1942); Reineke *et al.* (1945); Pitt-Rivers and Randall (1945). Fabre and Penau (1923) use permanganate for the oxidation of iodide to iodate and excess of permanganate is decomposed with alcohol. Michel and Lafon (1945) describe a method whereby the amount of iodine is converted to 36 times its original value. Iodate (obtained by bromine oxidation of iodide) is converted to iodine with KI in acid solution, extracted into CS_2, reduced with thiosulfate to iodide and re-oxidized to iodate.

2. Micro Methods

A. BASED ON THE HUNTER REACTION

The determination of iodine in blood, water, fats, hay, etc., as described by Harvey (1935), is based on the Hunter principle—alcohol is used for the extraction of iodide after alkaline incineration of the organic material. A detailed study of the purification of reagents for this assay has been made. Leipert (1933, 1934), Trevorrow and Fashena (1935), and Matthews *et al.* (1938) use chromium trioxide for the destruction of organic material. Riggs and Man (1940) use $KMnO_4$ and sulfuric acid.

B. THE COLORIMETRIC DETERMINATION OF IODINE

McClendon (1928) and co-workers (1929, 1930, 1931) describe methods for combustion of organic material followed by the colorimetric determination of iodine in carbon tetrachloride solutions. Talbot *et al.* (1944) and Koenig and Gustavson (1945) have used the starch iodine reaction for the colorimetric determination of iodine.

C. CERIC-ARSENITE REDUCTION METHOD FOR THE DETERMINATION OF IODINE

The iodine-containing organic material (blood, serum, thyroid, etc.) is destroyed with acid, and the iodic acid formed is reduced to iodine and distilled into alkali; the iodine is determined by its catalytic action on the rate of reduction of ceric sulfate by arsenious acid.

Chaney (1940), Strickler and Strickler (1945), Taurog and Chaikoff (1946b, 1948), and Barker (1948) describe modifications of this method.

Salter and Johnston (1948) (see also Salter and McKay, 1944) have described a simplified method which omits the distillation of iodine. Organic material is ashed with alkali, and the residue is dissolved in water. The iodide is determined by the ceric-arsenite method after acidification of the solution.

D. MISCELLANEOUS

An electrometric method for the determination of iodine in blood has been described by Paal and Motz (1935a); the same authors (1935b) have extended the method to the determination of thyroxine iodine. Harlay (1947) has described a fluorimetric method for the determination of iodine.

IV. DISCUSSION

1. The Chemical Assay of Thyroxine in the Thyroid Gland

Before discussing the chemical methods of thyroid assay which are used to elucidate problems in research, the methods used for the stand-

ardization of commercial thyroid preparations will be considered, since the theoretical considerations on which they are based have a direct bearing on the subject. Two such methods are in use today: assay by the determination of total iodine content, which is recommended by the United States Pharmacopoeia (1947) and by the French Codex (1937), and assay by the determination of acid-insoluble (thyroxine) iodine content, recommended by the British Pharmacopoeia (1948). Evidence can be brought forward in support of both methods. Gaddum and Hetherington (1931) found that the biological activity of desiccated thyroid preparations as measured by their effect on the metabolism of mice after oral administration was more closely related to the total iodine content than to the acid-insoluble iodine content (determined by the method of Harington and Randall, 1929b). Means *et al.* (1933) also came to the conclusion that the calorigenic activity of thyroid gland was proportional to the total iodine content and not the thyroxine iodine content, as tested by its action on human myxedema. Many workers (Thompson *et al.* 1929; see also Harington, 1933, p. 123) have noted the slow response of animals to injected thyroxine, and the oral administration of thyroxine to rats has been found less effective than that of thyroglobulin (Gaddum 1929–30). On the other hand, of the iodine-containing compounds of the thyroid, thyroxine alone possesses biological activity; diiodotyrosine possesses none when tested on mammals, and its activity when determined by the tadpole metamorphosis test is exceedingly small. On theoretical grounds therefore it is difficult to understand why there should not be a direct proportionality between thyroxine content and biological activity. A knowledge of the nature of the circulating thyroid hormone might throw light on this problem, but again the evidence is conflicting. Some workers hold that the thyroid hormone is in protein combination after its release from the gland into the blood stream. Riggs *et al.* (1942) found that it could not be removed from the serum proteins by dialysis and was precipitated with the serum proteins by the action of ordinary protein precipitants; further, this protein-bound iodine, when subjected to ultracentrifugation, was sedimented at a rate almost equal to that of albumin. Recently Salter and Johnston (1948) have examined the behavior of serum iodine toward acetone precipitation at different pH. The serum of a hyperthyroid patient was compared with the sera of normal and myxedema patients after both the latter had been made artificially hyperthyroidal by the addition of free thyroxine. At pH 3.5, acetone largely precipitated the iodine (natural hormone) from the true hyperthyroid serum but left the "artificial" hormone (added thyroxine) in solution in the normal and myxoedema sera. At pH

8.5 the hormone iodine (natural and artificial) was precipitated from all three sera. The authors infer from the results obtained at pH 3.5, which they regard as the most significant, that the natural hormone is not free thyroxine. On the other hand, there is much evidence from different sources for the opposite view.

Trevorrow (1939) found that 80% of the iodine in the blood of animals on an iodine-poor diet was organic, but was not in protein combination. It could be separated without previous hydrolysis from the blood proteins by butanol fractionation, as added thyroxine could be. Trevorrow concluded that the iodine in blood had thyroxine-like properties.

Lerman (1940) prepared thyroglobulin antisera in rabbits, with which he was able to detect the presence of minute amounts of thyroglobulin by the precipitin test; with their aid he was able to demonstrate the absence of thyroglobulin from the blood of many normal, myxedematous, and thyrotoxic patients and concluded that thyroxine or thyroxine-like compounds were "the true accelerators of metabolic function."

Harington (1944) reviewed the immunological evidence with regard to the nature of the thyroid hormone in blood, based on his own (Clutton et al., 1938) and other work; this, together with other evidence of a more general nature led him to conclude that thyroxine is present in the free state in blood. This theory has recently been put forward again by Taurog and Chaikoff (1947–48) who have produced very strong supporting evidence based on their observations concerning the nature of plasma iodine; their evidence will be briefly summarized. (1) Ninety per cent of plasma iodine was extractable into n-butanol, and only 10% of the extracted iodine was removed by alkaline washing. (None of the thyroid iodine behaved in this manner until the gland had been hydrolyzed.) (2) If thyroxine were added to plasma, 80% of it could be recovered by butanol extraction. (3) Thyroxine added to plasma was non-dialyzable and was precipitated together with the plasma proteins by zinc hydroxide. Experiments were also done using radioactive iodine, with the following results. (4) Twenty-four hours after the administration of I^{131} to rats, 90% of the radioactivity in the plasma was protein-bound and 72% was butanol-extractable. (5) If inactive thyroxine were added to the butanol extracts of I^{131} and isolated, it had constant activity after repeated recrystallization. (6) Carrier thyroxine and I^{131} from plasma were partitioned between aqueous alkali and organic solvents (n-butanol and iso-amyl alcohol). The distribution of radioactive I always paralleled that of the carrier thyroxine. (7) A thyroxine peptide, prepared according to Harington and Salter (1930)

was also partitioned with I^{131} between immiscible solvents. The distribution of the thyroxine peptide carrier was quite different from that of the plasma I^{131}.

On the whole, the balance of evidence indicates that the thyroid hormone is liberated from the gland, circulates in the blood and acts on the peripheral tissues as free thyroxine. It follows that the thyroxine content of any thyroid preparation should be the best index of its activity.

The methods of determining thyroxine in the thyroid will now be considered. Harington and Randall's (1929b) method which involves the hydrolysis of thyroid preparations for 4 hours with 10 parts of N NaOH has been criticized by many workers on the grounds that the time of hydrolysis is inadequate and leads to high thyroxine values being found; further, Doery (1945) has shown that the actual amount of alkali used in the Harington and Randall hydrolysis is insufficient—this has been confirmed by Pitt-Rivers (unpublished work) who has found that in the Harington and Randall estimation, an increase in the amount of alkali from 10 to 50 parts reduces the thyroxine iodine value by 15%. It is of course possible that the decrease in thyroxine iodine might be due to losses through solubility of incompletely hydrolyzed thyroxine peptides. Nevertheless, the bulk of evidence does indicate that the Harington and Randall method gives high and not easily reproducible results, and it is concluded that any standard method of thyroxine iodine assay should observe the following conditions: (1) a longer period of hydrolysis, (2) stronger alkali, and (3) a more dilute solution of the protein. If changes on these lines are made, this simple chemical method of thyroid assay is theoretically valid for the determination of the potency of thyroid preparations, and the more complicated methods need not be considered in this connection. For the assay of very small amounts of thyroxine, i.e., in single thyroids of small animals, a more elaborate technic is required for separating thyroxine from other iodine-containing compounds (diiodotyrosine, iodide) since the limit of insolubility of thyroxine will be reached. Kendall (1915) showed that a part of the acid-insoluble fraction from a thyroid hydrolysate could be extracted into ethyl acetate, but Blau (1933) found that this solvent was not satisfactory and substituted n-butanol for the acid extraction of thyroxine. This solvent had already been used by Leland and Foster (1932) for the extraction of thyroxine from an alkaline aqueous phase, and the distribution ratios of thyroxine and diiodotyrosine would seem to favor this procedure. Diiodotyrosine is extracted from an aqueous phase at pH 3–4 to the extent of 17% whereas it is only extracted from $2N$ NaOH to the extent of 2.5% (Leland and Foster). However Blau showed that one re-extraction of the acid butanol extract with $4N$ NaOH

containing 5% sodium carbonate, removed 95% of the diiodotyrosine. Blau's findings have been confirmed by other workers (Reineke *et al.*, 1945; Taurog and Chaikoff, 1946) in recovery experiments on known mixtures of thyroxine and diiodotyrosine and Blau's method for the separation of these two amino acids is the one now most generally used.

For the determination of quantities of thyroxine between 0.1 and 1 mg. the colorimetric methods of Roche and Michel (1947) and of Winikoff and Trikojus can be used. The color reaction of Roche and Michel (Kendall-Osterberg) is non-specific and therefore depends upon an unequivocal separation of thyroxine from diiodotyrosine. The color reaction of Winikoff and Trikojus for thyroxine is not given by diiodotyrosine, and the method is therefore to be preferred to the former.

For the determination of quantities of thyroxine of the order of 2 μg. or less, micro methods of iodine assay must be used. Reference has been made to a number of these in Section III; in Harvey's method (1935), the iodine is determined by $N/500$ sodium thiosulfate; in that of Matthews *et al.* (1938), $N/1000-N/5000$ thiosulfate is used. The most recent methods for the micro determination of iodine are based on Chaney's (1940) ceric-arsenite reduction method and in some (Taurog and Chaikoff, 1946b; Salter and Johnston, 1948) fractions of 1 μg. of iodine have been determined with a reasonable degree of accuracy. Taurog and Chaikoff have controlled their method of iodine determination with the aid of radioactive iodine.

The assay of thyroxine by means of thyroxine iodine determinations, or by the use of other non-specific reactions, is only justifiable when there is a reasonable degree of certainty that the "thyroxine" fraction does in fact contain thyroxine alone. This applies to biological materials such as blood, thyroid, and other tissues since there is no evidence at present of the existence of any naturally occurring compound with chemical or physical properties similar to those of thyroxine which would interfere with the thyroxine assay by any of the methods given above.

2. The Chemical Assay of Artificial Thyroproteins

On theoretical grounds, the chemical assay of artificial thyroproteins presents a much more complex problem than that of the natural hormone, since the constitution of many of the iodine-containing compounds formed during iodination of proteins is still unknown. So far, only four of these compounds have been isolated and characterized: monoiodotyrosine, diiodotyrosine, thyroxine, and 3,5-diiodo-4-hydroxybenzaldehyde (Ludwig and von Mutzenbecher, 1939; Reineke and Turner, 1943; Pitt-Rivers, 1948, etc.) and of these, thyroxine alone is active; the rest

of the uncharacterized iodine-containing compounds are associated with thyroxine by virtue of their physical properties (acid-insolubility; butanol solubility); they differ fundamentally from thyroxine in possessing (so far as is known at present) little or no biological activity (Blaxter, 1945; Deanesly and Parkes, 1945). This inactivity is of course ascribed collectively to the acid-insoluble group of compounds other than thyroxine: the individual activity of any one component of the group can only be determined at such a time when it is isolated and characterized. In this connection, some recent work of Hird and Trikojus (1948) is of particular interest; these authors examined chromatographically the acid-insoluble fraction of an alkaline hydrolysate of a preparation of iodinated casein; the examination revealed the presence of thyroxine and diiodothyronine (trace); a third amino acid appeared on the chromatogram in a position between thyroxine and diiodothyronine, and. the authors suggest that it might be 3,3',5-triiodothyronine which has not hitherto been described. Chromatographic analysis of a crude crystalline by-product obtained during the purification of the thyroxine fraction revealed the same three iodine-containing amino acids; the origin of two of them (diiodothyronine and triiodothyronine) was speculated on by the authors, and for various reasons they concluded that they were not artefacts of hydrolysis, but had been formed during iodination by the oxidative coupling of tyrosine and monoiodotyrosine with diiodotyrosine (giving di- and triiodothyronine respectively). The crude by-product mentioned above possessed about one-third of the biological activity of thyroxine and contained 75% of thyroxine by colorimetric assay. Hird and Trikojus point out that triiodothyronine might well be included in such an assay so that the thyroxine assay is not therefore significant. Whether any of the biological activity of the whole fraction was due to the hypothetical triiodothyronine cannot at present be more than a conjecture, although the known activity of 3,5-diiodothyronine (Anderson, Harington, and Murray Lyon, 1935) is suggestive. Nor does our present knowledge concerning the mechanism of thyroxine formation warrant more than the most tentative suggestions as to the coupling of dissimilar tyrosine or iodinated tyrosine molecules or the unsymmetrical coupling of similar or dissimilar molecules, e.g., such as could lead to Niemann and Mead's (1941) biologically active isomer of thyroxine. At present, this is all hypothesis, and until more facts are discovered, we must assume that the biological activity of iodinated proteins is due to thyroxine alone.

With this assumption in mind, the specificity of the different methods of chemical assay as applied to artificial thyroproteins will be discussed.

In the methods where "thyroxine" assay is non-specific, i.e., iodine

determination or certain colorimetric reactions, it is essential that hydrolysis of the protein should be complete so that the thyroxine can be separated from interfering compounds; the method of hydrolysis is therefore of the first importance. Prolonged hydrolysis of the protein with 2N NaOH was recommended by Leland and Foster (1932) who found that the thyroxine iodine, expressed as a percentage of total iodine, increased rapidly during the first 6–8 hr. of hydrolysis after which it maintained a fairly constant value up to 20 hr. Taurog and Chaikoff (1946a), who favor the Leland and Foster method of hydrolysis have confirmed that the thyroxine iodine does not greatly decrease between 7 and 20 hr. hydrolysis. Reineke *et al.* (1945), Simpson *et al.* (1947), Borrows *et al.* and Pitt-Rivers (1949) prefer 40–50% baryta hydrolysis (see Ludwig and von Mutzenbecher, 1939) to NaOH hydrolysis. On theoretical grounds it would appear to be less destructive to thyroxine since the latter (Harington, 1926a) is removed during hydrolysis as an insoluble barium salt from further action of the alkali.

Blau (1935) proposed that a short hydrolysis (6 hr. with 8% baryta) should be substituted for Leland and Foster's (1932) prolonged NaOH hydrolysis and claimed that the method led to higher thyroxine values being obtained. Blau's hydrolysis has been adopted by Roche and Michel (1947) in their method for the determination of thyroxine in iodinated proteins, and the separation of thyroxine is effected by acid-butanol extraction as first described by Blau. Now a mild hydrolysis such as this may not liberate thyroxine completely from peptide linkage, in which case, the effective separation of thyroxine will depend upon the thyroxine-like or diiodotyrosine-like properties of the peptide residues present, and the presence of mono- or diiodotyrosine in the thyroxine peptides may well mask its characteristic properties. Harington (1926a) in his isolation of thyroxine from the thyroid obtained two crude thyroxine fractions (as insoluble barium salts and as an acid-insoluble fraction) after the preliminary short hydrolysis with 10% baryta; both fractions required further prolonged hydrolysis with 40% baryta before thyroxine was isolated in a relatively pure condition. Pitt-Rivers has attempted without any success to isolate crystalline thyroxine from an active iodinated casein preparation by dilute baryta hydrolysis (unpublished work). Reineke *et al.* (1945) and Taurog and Chaikoff (1946a) have reported that variable results in thyroxine values are obtained if thyroid or thyroprotein is hydrolyzed by Blau's method. This evidence indicates that the concentration of baryta or time of hydrolysis should be increased in order to effect complete liberation of the thyroxine in proteins.

The fractionation of thyroxine into *n*-butanol must also be commented on. It has been seen that Borrows, Hems, and Page have questioned

the values previously given for the distribution coefficient of diiodo-
tyrosine in butanol NaOH mixtures; the problem of the separation of
diiodotyrosine has, however, been settled by the acid-butanol extraction
technic.   A far more serious objection to butanol fractionation (either
from acid or alkaline solution) as an effective means of separating thyrox-
ine from interfering substances, is our almost complete ignorance of the
nature and properties of many of the compounds formed during iodina-
tion of proteins.   Pitt-Rivers (1948) showed that butanol extraction of
the NaOH hydrolysate of a relatively inactive sample of thyroprotein
removed 50% of the acid-insoluble iodine; of this, less than 10% repre-
sented thyroxine iodine (as determined by isolation); 92% of it was con-
tained in uncharacterized by-product.   The determination of thyroxine
as "butanol-soluble iodine" is not therefore justifiable for the assay of
thyroproteins.   .
    These remarks apply equally to other non-specific methods for
thyroxine estimation; indeed, they apply also to the methods which have
been shown to be specific for thyroxine and diiodotyrosine only, since
their specificity cannot have been determined as regards impurities
whose constitution is not yet determined.   Saul and Trikojus (1948)
have demonstrated that the lactic acid analog of thyroxine gives the
same color as thyroxine itself with the thyroxine reagent of Winikoff
and Trikojus (1948).   It is probable that any tetraiodohydroxydiphenyl
ether possessing the same orientation of iodine atoms as thyroxine will
be included in "thyroxine" which is assayed by this method.   Borrows
et al. have made the same comment with regard to polarographic analysis
and the determination of thyroxine from absorption spectra data is also
open to the same objection; indeed, Reineke and Turner (1942) suggested
that the absorption spectra curves of excessively iodinated proteins
possessing low biological activity might be due to the presence of inactive
compounds such as Bovarnick's *et al.* (1939) inactive analog of thyroxine
which has three diiodophenolic residues.
    At the present time, there is no chemical method for the assay of
thyroxine in artificial thyroproteins which will lead to unambiguous
results.   Pitt-Rivers (1949) has proposed that thyroxine should be
assayed by isolation of the pure amino acid and considers that this is
the only means of determining thyroxine alone, that is, excluding the
unknown inactive contaminants.   The method described by Pitt-Rivers
is rather crude and has the disadvantage of requiring large amounts of
material for assay, when compared with the method of Roche and Michel
(1947) or Borrows *et al.;* further, the method is open to the criticism that
serious losses of thyroxine may occur during isolation when the amount
of thyroxine present in the protein is small compared with the amount

of contaminating impurities, since crystallization of thyroxine under such conditions is difficult to achieve. Michel and Pitt-Rivers (1948) attempted the assay of iodinated silk fibroin preparations by an isolation method; in one instance only 2 mg. of thyroxine was obtained from about 5 g. of the protein, and it is quite probable that losses of thyroxine may have led to an error in the determination of 100% or more. Refinements in the technic of isolation, e.g., butanol extraction, are indicated, before any degree of accuracy can be claimed for this method of thyroxine assay; as it stands, it will only give a rough quantitative measure of the amount of hormone present in artificial thyroproteins.

The application of two relatively new technics toward the solution of the problem of thyroxine assay is worth considering. Many problems concerning the metabolism of iodine have until recently eluded solution because the technics available for the detection and determination of very small quantities of iodine in biological material were inadequate. The use of radioactive iodine has now made possible the investigation of these problems, and much work has been done with its aid. In particular, the work of Chaikoff and of Leblond and their collaborators (reviewed by Hevesy, 1948) on the uptake of iodine by the thyroid, its conversion into the thyroid hormone, its nature and distribution in the tissues, and its metabolism has contributed most to our present knowledge on the subject. It may well be that methods which involve the use of radioactive iodine or labeled thyroxine (Joliot, 1945) will be devised for the study of such problems as the loss of thyroxine during purification.

The behavior of the iodinated amino acids on paper chromatograms has been studied by Dent (1948) and others (Fink *et al.*, 1947; Fink and Fink, 1948, etc.). So far the investigations of these workers have been of a qualitative nature, and it is not possible to predict at present how far the technic of paper partition chromatography will be applicable to quantitative problems. Preliminary studies toward the chromatographic separation of iodinated impurities which are formed together with thyroxine during the iodination of proteins have been made by Hird and Trikojus (1948) which may eventually lead to the complete separation and identification of these products. If this is done, the biological activity of individual components can be determined and any active components will have to be included in the chemical assay; until this has been done, only the determination of thyroxine can give a chemical measure of the biological potency of products possessing thyroidal activity.

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